(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
25 September 2003 (25.09.2003)

PCT

(10) International Publication Number
WO 03/077837 A2

(51) International Patent Classification[7]: A61K

(21) International Application Number: PCT/US02/39332

(22) International Filing Date:
10 December 2002 (10.12.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/363,239 11 March 2002 (11.03.2002) US

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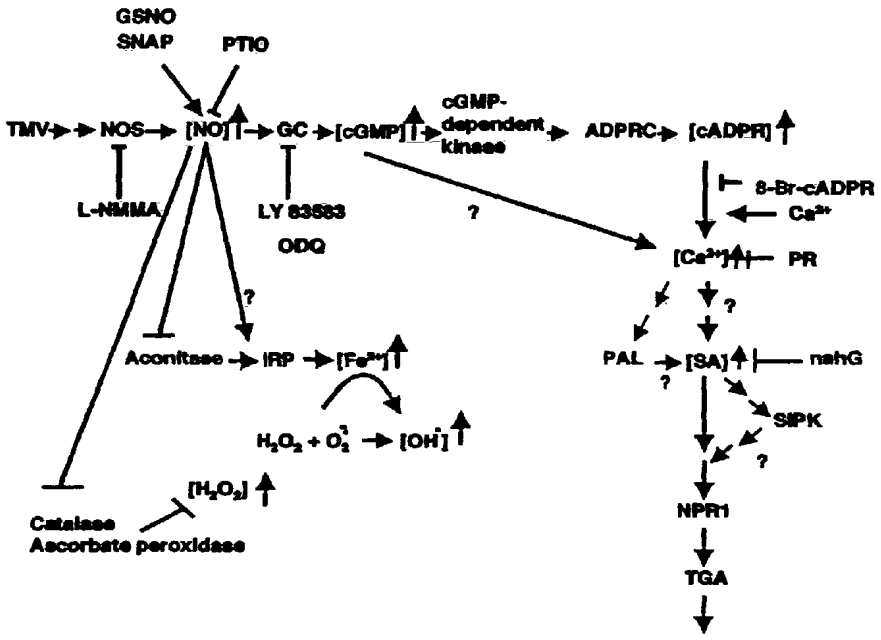
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SI, SK,

[Continued on next page]

(54) Title: PLANT NITRIC OXIDE SYNTHASE



(57) Abstract: A plant nitric oxide synthase (NOS) is disclosed. The enzyme isolated from tobacco possesses numerous features characteristic of animal NOSs. The deduced amino acid sequence of the corresponding protein from Arabidopsis is also disclosed. The Arabidopsis protein possesses NO-synthesizing activity and, as in tobacco, its activity is induced by infection with avirulent pathogens.

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TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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PLANT NITRIC OXIDE SYNTHASE

This application claims benefit of U.S. Provisional Application No. 60/363,239, filed March 11, 2002, the entirety of which is incorporated by reference herein.

FIELD OF THE INVENTION

This invention relates to the field of plant physiology and molecular biology. In particular, the invention provides a novel plant nitric oxide synthase and methods for its use.

BACKGROUND OF THE INVENTION

Various patents and other publications are referenced herein. These patents and publications are incorporated by reference in their entireties.

In plant-pathogen interactions, a susceptible plant cannot effectively restrict pathogen growth and/or spread. Thus, the pathogen often causes severe damage or even death of the entire plant. In contrast, a resistant plant is capable of deploying a variety of defense responses to prevent pathogen colonization. A key difference between resistant and susceptible plants is the timely recognition of the invading pathogen and the rapid and effective activation of host defenses. Plants resisting pathogen attack often exhibit a wide range of physiological changes, including the rapid production of large quantities of reactive oxygen species (ROS; termed an oxidative burst), transient ion-fluxes leading to intracellular pH changes, protein phosphorylation, cell wall strengthening near the infection site(s) and the synthesis of

antimicrobial products including phytoalexins and pathogenesis-related (PR) proteins. Activation of resistance also is frequently manifested by a hypersensitive response (HR), in which necrotic lesions develop at the sites of infection (this necrosis has many characteristics which resemble animal programmed cell death) and pathogen growth and spread is restricted. This cell death response is likely to benefit the plant by depriving the pathogen access to nutrients and limiting its ability to proliferate. In addition, signal molecules produced by the dying cells activate a variety of defense-related genes.

In addition to the localized defense response, many plants respond to pathogen infection by activating a subset of defenses in the uninfected parts of the plant. Over a period of hours to days after the primary infection, systemic acquired resistance (SAR) develops throughout the plant. SAR is manifested as an enhanced and long-lasting resistance to secondary challenge by the same or even unrelated pathogens; it shares many similarities with innate immunity in vertebrates and invertebrates.

In general, a plant's reaction to biotic and abiotic stresses requires the perception of a stimulus by a receptor and the subsequent involvement of second messengers and effector proteins to trigger an appropriate response. The activation of plant defense mechanisms is frequently initiated by host recognition of race-specific (e.g. avirulent gene products) or non-specific signals (elicitors) derived from or generated by the invading pathogen (e.g. microbial proteins, glycoproteins, small peptides and oligosaccharides, etc.). Recognition of these molecules may be mediated in the extracellular spaces by a membrane-bound receptor, as has been shown for certain bacterial and fungal elicitors. Alternatively, in some gene-for-gene interactions, an avirulence gene product (e.g. AvrPto or AvrPi-ta) may enter the plant

cell and interact directly with a cytosolic receptor encoded by the corresponding disease resistance (R) gene. Following the initial perception event, resistance-associated signals may be transduced through G proteins, ion fluxes, ROS, and/or phosphorylation cascades involving various kinases/phosphatases, including mitogen-activated protein (MAP) kinases. Among the signaling molecules or hormones involved in plant defense reactions are salicylic acid (SA), jasmonates, ethylene, nitric oxide (NO) and, perhaps, the peptide systemin. Subsequent transcriptional and/or post-translational activation of transcription factors eventually leads to the induction of plant defense genes.

NO is produced during the conversion of L-arginine to citrulline by a family of enzymes termed NOS (nitric oxide synthase). These enzymes are usually referred to as 'dimeric' in their active form; however, since calmodulin (CaM) also is required for activity, NOSs technically are tetramers of two NOS monomers and two CaMs. They contain several tightly-bound cofactors, including heme, FAD, FMN and tetrahydrobiopterin (H_4B), and catalyze the reaction of L-arginine, NADPH, and O_2 to NO, citrulline and NADP. In mammals, three distinct isoforms of NOS have been identified; they represent the products of three different genes, with 51–57% homology between the human isoforms.

In addition to mammals, there is a growing interest in NO production in other species. NOS activity has been described in organisms as simple as a single cell slime mold to more complex animals such as insects, mollusks, echinoderms, fish, amphibians, reptiles and birds. In non-mammalian vertebrates, NADPH-d histochemical or NOS immunocytochemical techniques have demonstrated NOS in the central nervous system of various species including fish, amphibians, reptiles, and

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birds. In contrast to the above, neither the enzyme itself nor a gene with obvious homology to the mammalian NOS-encoding genes has been isolated from plants. However, NOS-like activities have been reported in TMV-infected tobacco (Durner, J., et al., 1998, Proc. Natl. Acad. Sci. USA 95: 10328-10333.), elicitor-treated soybean cells (Delledonne, M., et al., 1998, Nature 394: 585-588.), peroxisomes of pea (Barroso, J.B., et al., 1999, J. Biol. Chem. 274: 36729-36733; Corpas, F.J., et al., 2001, Trends Plant Sci. 5: 145-150), maize seedlings (Ribeiro, E.A., et al., 1999, FEBS Lett. 445: 283-286), roots of Lupinus albus (Cueto, M., et al., 1996, FEBS Lett. 398: 159-164.) and *Arabidopsis* (Mackerness, S.A.-H., et al., 2001, FEBS Lett. 489: 237-242).

Pathogen attack and environmental stress are major factors limiting plant growth and productivity. Many chemicals have been developed to help prevent crop disease and control herbivorous pests, but they are costly and can be toxic to the environment and people. Moreover, the benefit of agrochemicals is often overcome relatively quickly by rapid evolution of plant pathogens. Engineering disease and pest resistance in plants can provide environmentally friendly and healthier alternatives to these traditional chemical methods of agriculture. A variety of alternative strategies are being developed to protect plants against disease. Currently, resistance to several viruses has been engineered by transforming susceptible plants with genes or sequences derived from the respective viral genomes (pathogen-derived resistance); some of these crop plants are already in use by farmers. Another approach involves generating transgenic plants that synthesize antimicrobial enzymes or products (e.g. PR-1, b-1,3-glucanase, chitinase, ribosome inactivating proteins, thionins, defensins, or the phytoalexin resveratrol). A third strategy to control disease is the use of R

genes, a large number of which have been cloned. These include the N gene of tobacco, which confers resistance to TMV. However, with all the above approaches, protection is generally conferred against just one or a few pathogens, or only a specific strain of a given pathogen.

An alternative approach is to induce the plant's own natural defenses. This strategy is attractive since it can provide protection against a broad spectrum of pathogens. Broad-spectrum resistance to a wide array of pathogens can be activated by infection with a SAR-inducing pathogen or treatment with a SAR-inducing chemical such as SA, 2,6-dichloroisonicotinic acid (INA) or benzothiadiazole (BTH). INA and BTH promote disease resistance by acting as functional analogs of SA. However, SA and INA are either not effective enough at low concentrations or too toxic at high levels to be utilized successfully in the field. By contrast, BTH has been shown to enhance resistance in several crop species against various pathogens. A major caveat to this approach is that the SAR-inducing compounds work only if applied prior to pathogen infection. In addition, pre-inoculating crops with an SAR-inducing pathogen runs the risk of cross-infecting nearby susceptible plants.

A more successful strategy for enhancing disease resistance will therefore involve engineering plants that can more rapidly or effectively activate their defenses upon attack. (Note that constitutively activated SAR generally appears to impose a significant fitness penalty, which results in stunted or morphologically abnormal plants). It is currently thought that pathogen perception and the first few signaling steps are distinct for each plant-pathogen interaction. However, these signals then appear to converge on a limited set of defense pathways. Thus, identification and characterization of the components involved in these common pathways, such as NOS

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and NO effector proteins, should provide an opportunity to manipulate resistance to a broad spectrum of pathogens. Consistent with this notion, over-expression of the defense signal transducers Npr1, Pfr or Pto induces broad-spectrum resistance in *Arabidopsis* and tomato.

SUMMARY OF THE INVENTION

This invention provides a novel plant nitric oxide synthase (NOS), which is involved in one or more signal transduction pathways leading to disease defense responses in plants.

In one aspect of the invention, an isolated plant enzyme having nitric oxide synthase (NOS) activity is provided. The enzyme is referred to herein as piNOS (plant inducible nitric oxide synthase). The NOS activity of piNOS is inducible in a plant and comprises production of nitric oxide from arginine. Loss of the NOS activity of the enzyme in a plant is expected to result in altered resistance of the plant to plant pathogens or other disease-causing agents. The NOS activity of the enzyme is characterized by one or more of the following features: (a) having activity that is inducible by a plant pathogen; (b) having activity dependent on H_4B, FAD, FMN and NADPH; (c) requiring Ca^{2+} for activity; (d) requiring calmodulin for activity; and (e) synthesizing NO as determined by a citrulline formation assay or by an oxy-hemoglobin assay. The enzyme is further characterized by one or more of the following features: (a) activity inhibited by L-NMMA; (b) activity inhibited by aminoguanidine; (c) exhibiting calmodulin-mediated change in flavin fluorescence; and (d) exhibiting calmodulin-mediated change in tryptophan fluorescence. The enzyme isolated from tobacco has an apparent molecular mass of about 120 kDa as

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determined by SDS-PAGE. It may be isolated from tobacco inoculated with TMV, according to the following method: (a) obtaining a crude extract of TMV-inoculated tobacco leaves; (b) subjecting the crude extract to G-25 Sephadex chromatography and collecting a void volume obtained therefrom; (c) subjecting the void volume to ammonium sulfate precipitation and collecting the 30-35% of saturation ammonium sulfate precipitate therefrom; (d) subjecting the dissolved 30-35% ammonium sulfate precipitate to DEAE-Sepharose chromatography and collecting NOS activity-containing eluate therefrom; and (e) subjecting the NOS activity-containing eluate from the DEAE-Sepharose chromatography to Arg-Sepharose chromatography and collecting NOS activity-containing eluate therefrom. Further purification is accomplished by performing one or both of the following additional steps: (f) subjecting the NOS activity-containing eluate from the Arg-Sepharose chromatography to ADP-Sepharose chromatography and collecting NOS activity-containing eluate therefrom; and (g) subjecting the NOS activity-containing eluate from the ADP-Sepharose chromatography to CaM-Sepharose chromatography and collecting NOS activity-containing eluate therefrom.

Antibodies immunologically specific for part or all of the above described piNOS enzyme are also featured in the present invention. In one embodiment, the antibodies are immunologically specific for an amino-terminal portion of the enzyme, particularly the portion comprising the N-terminal 80-or-so residues.

In embodiments of the invention, the piNOS enzyme comprises an amino acid sequence that contains one or more fragments as follows:

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FGVPMGYGGPHAAFLATSQEYKR (SEQ ID NO:2);

GNINIEEVRK (SEQ ID NO:3);

IAILNANYMAK (SEQ ID NO:4); and

GNADVQNNVL (SEQ ID NO:5).

In one embodiment, the enzyme has amino acid SEQ ID NO:1, which is the deduced amino acid sequence of the *Arabidopsis* piNOS. In further embodiments, the enzyme is produced by expression of an isolated nucleic acid molecule encoding the enzyme.

According to another aspect of the present invention, an isolated nucleic acid molecule having a coding sequence that encodes the above-described piNOS enzyme is provided. In certain embodiments, the nucleic acid molecule encodes enzyme has an amino acid sequence comprising one or more of the following fragments of SEQ ID NO:2, NO:3, NO:4 and NO:5 as described above. In an exemplary embodiment, the nucleic acid molecule encodes amino acid SEQ ID NO:1.

In further embodiments the nucleic acid has at least 90% sequence identity with SEQ ID NO:6. In preferred embodiments, the nucleic acid molecule has at least 60% sequence identity with a portion of SEQ NO:6 encoding an approximately 80-residue amino-terminal portion of the piNOS enzyme. In further embodiments, the nucleic acid molecule hybridizes with the complement of SEQ ID NO:6 under stringent hybridization conditions comprising hybridization at least six hours at 42°C in 5X SSC, 5X Denhardt's reagent, 1.0% SDS, 100 μg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide; and washing at least once at room temperature in 2X SSC and 0.1 - 1% SDS with a final wash at 65°C in 0.1X SSC and 0.1% SDS for 10 minutes. In a preferred embodiment,

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the nucleic acid molecule hybridizes with the complement of a portion of SEQ ID NO:6 comprising an approximately amino-terminal portion of the enzyme, under moderate hybridization conditions comprising hybridization at least six hours at 42°C in 5X SSC, 5X Denhardt's reagent, 1.0% SDS, 100 μg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide; and washing at least once at room temperature in 2X SSC and 0.1 - 1% SDS with a final wash at 45-55°C in 2X SSC and 0.1% SDS for10 minutes. In an exemplary embodiment, the nucleic acid molecule comprises SEQ ID NO:6.

The aforementioned piNOS-encoding nucleic acid molecule may be a gene having an open reading frame that comprises the coding sequence. Alternatively, it can be a mRNA molecule produced by transcription of the gene or a cDNA molecule produced by reverse transcription of the mRNA molecule. Also provided in accordance with the invention is an oligonucleotide between 8 and 100 bases in length, which is complementary to a segment of the piNOS-encoding nucleic acid molecule.

In another aspect of the invention, vector comprising one or more of the above-described polynucleotides or oligonucleotides is provided. In particular embodiments, the vector is an expression vector that may be a plasmid, cosmid, baculovirus, bacmid, bacterial vector, yeast or other fungal vector, or viral vector. In one embodiment, the vector contains a piNOS the coding sequence operably linked to a constitutive promoter. In another, it is operably linked to an inducible promoter.

Host cells transformed with the vectors of the invention are also provided. These may be plant cells, bacterial cells, fungal cells, insect cells or mammalian cells.

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In one embodiment, the cell is a plant cell that may be obtained from any plant, including but not limited to tobacco, *Arabidopsis*, maize, wheat, rice, soybean barley, rye, oats, sorghum, alfalfa, clover, canola, safflower, sunflower, peanut, tomato tomatillo, potato, pepper, eggplant, sugar beet, carrot, cucumber, lettuce, pea, aster, begonia, chrysanthemum, delphinium, zinnia, turfgrasses. Fertile plants produced from the transformed plant cells are also featured in accordance with an aspect of the invention.

Another aspect of the invention features kits for producing transgenic host cells that produce a piNOS enzyme. Such kits contain an appropriate vector as described above, along with instructions for producing the transgenic cells using the vector. The kits may also contain one or more additional components, such as culture media for culturing the cells, reagents for performing transformation of the cells, reagents for testing the transgenic cells for piNOS-encoding gene expression, and reagents for testing the transgenic cells for piNOS enzymatic activity.

According to another aspect of the invention, methods for identifying agents that modulate piNOS enzymatic activity are provided. These methods comprise: (a) combining a pre-determined amount of the piNOS with a substrate acted upon by the piNOS to produce a product, wherein the substrate or the product are directly or indirectly detectable, the combining being performed in the presence or absence of a test compound suspected of modulating the piNOS activity; and (b) measuring conversion of the substrate to the product in the presence and absence of the test compound, a change in rate or total amount of conversion of the substrate to the product in the presence of the test compound, as compared to in the absence of the test compound, being indicative that the test compound modulates piNOS enzymatic

activity. In one embodiment, the piNOS is affixed to a solid support. In another embodiment, the method is adapted for identifying agents that modulate piNOS enzymatic activity in host cells, and comprises the steps of (a) introducing a piNOS-encoding nucleic acid molecule into the host cells under conditions whereby piNOS is produced by expression of the nucleic acid molecule; (b) treating a sample of the host cells with a test compound suspected of modulating the piNOS enzymatic activity; and (c) comparing piNOS enzymatic activity in the sample of host cells treated with the test compound with piNOS enzymatic activity in an equivalent sample of host cells not treated with the test compound, a difference between the comparative piNOS enzymatic activities being indicative that the test compound modulates piNOS enzymatic activity.

According to yet another aspect of the invention, methods for identifying agents that modulate expression of genes encoding piNOS in host cells are provided, comprising the steps of (a) introducing into the host cells a piNOS-encoding gene comprising a *piNOS* coding region and *piNOS* expression regulatory sequences; (b) treating a sample of the host cells with a test compound suspected of modulating genes encoding piNOS; and (c) comparing piNOS-encoding gene expression in the sample of host cells treated with the test compound with piNOS-encoding gene expression in an equivalent sample of host cells not treated with the test compound, a difference between the comparative piNOS-encoding gene expressions being indicative that the test compound modulates expression of genes encoding piNOS. The piNOS-encoding gene expression can be measured in a variety of ways, including measuring the amount of piNOS-encoding mRNA or piNOS protein, or by measuring piNOS enzymatic activity in the treated and untreated cells. In an alternative method,

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the piNOS coding sequence is replaced by a heterologous coding sequence encoding a detectable gene product and gene expression is measured by measuring the detectable gene product.

Yet another aspect of the invention features a method to enhance resistance of a plant to plant pathogens or other disease causing agents. The method comprises increasing an amount or activity in the plant of a piNOS enzyme having nitric oxide synthase (NOS) activity, wherein the NOS activity is inducible in a plant and comprises production of nitric oxide from arginine. In one embodiment, the method comprises over-expressing a gene encoding the piNOS enzyme in the plant, which may be accomplished by introducing a piNOS-encoding transgene into the plant, as well as by other methods. In another embodiment, piNOS activity is enhanced by treating the plant with a modulator of piNOS enzymatic activity identified by one of the methods set forth herein. In another embodiment, piNOS-encoding gene expression is further enhanced by treating the plant with a modulator of piNOS-encoding gene expression identified by the methods set forth herein, of a type that increases expression of genes encoding piNOS.

Another aspect of the invention features a method of inhibiting expression of a piNOS-encoding gene in a plant, which comprises introducing a nucleic acid molecule into the plant that inhibits the expression of the plant's piNOS-encoding gene. In one embodiment the nucleic acid encodes a molecule that is antisense to the plant's piNOS-encoding genes, such as represented by SEQ ID NO:6 in *Arabidopsis*. In another embodiment, the nucleic acid comprises a double stranded RNA segment of a piNOS-encoding gene, preferably containing part or all of an intron. In yet another embodiment, the nucleic acid comprises a piNOS-encoding sequence and

inhibition of expression the plant's piNOS-encoding gene occurs through co-suppression by the introduced nucleic acid.

According to another aspect of the invention, a method to inhibit piNOS-mediated signal transduction in a plant is provided. This method comprises introducing into the plant a mutated piNOS-encoding nucleic acid, which encodes a nonfunctional piNOS enzyme.

Transgenic plants having enhanced resistance to plant pathogens or other disease-causing agents are also provided in accordance with another aspect of the invention. The plants comprise a transgene that encodes a plant enzyme having nitric oxide synthase (NOS) activity, wherein the NOS activity is inducible in the plant and comprises production of nitric oxide from arginine. In one embodiment, the transgene encodes SEQ ID NO:1. Fertile transgenic plants are preferred for some uses.

Also provided in accordance with an aspect of the invention are transgenic knock-out plant, wherein expression or activity of the plant's endogenous piNOS enzyme is absent or reduced as compared to a non-transgenic plant of the same type. Fertile transgenic plants of this type are preferred for use in some embodiments.

Other features and advantages of the present invention will be understood by the drawings, detailed description and examples set forth herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A. NOS activities in the extracts of mock or TMV-infected tobacco leaves after temperature shift. Xanthi nc (NN) plants were infected with TMV (Δ) or buffer (mock) (●) while Xanthi (nn) was infected with TMV (❑). Defense

responses were activated by shifting from 32°C to 22°C, 48 hr after infection. At 32°C defense responses to TMV in Xanthi nc (NN) are suppressed, but upon shifting to lower temperatures (<28°C) these responses are rapidly activated (Durner et al., 1998). The time plotted on the x-axis is in hr after shift to 22°C. NOS activity was measured at 401 nm, using oxy-hemoglobin assay. The data represent the mean of three independent experiments with each time point in each experiment done by monitoring NOS activity in triplicate. Thus, each data point above represents the average of nine assays.

Fig. 1B. NOS activities in extracts of tobacco leaves after TMV infection at 22°C in Xanthi nc (NN). Plants were infected with TMV and the tissue was frozen at a different time after infection; NOS activity was measured at 401 nm using oxy-hemoglobin assay. The data represent the mean of three independent experiments as above.

Fig. 2. NOS activity in the extracts of mock and *Pseudomonas syringe* pv. Maculicola infected tobacco leaves. Xanthi nc (NN) leaves were inoculated with *P. syringae* pv maculicola or magnesium chloride (mock) at 22°C and the tissue was frozen at various points after infection. NOS activity was measured at 401 nm using oxy-hemoglobin assay. The data represent the mean of three experiments as in Fig. 1A.

Fig. 3. Fractionation of tobacco NOS activity by DEAE-Sepharose chromatography. The void volume fraction from G-25 Sephadex chromatography, which contained the majority of the NOS activity, was subjected to differential ammonium sulfate precipitation. The 30-35% ammonium sulfate precipitate was

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dissolved in equilibration buffer and loaded onto a DEAE-Sepharose column and allowed to bind. After washing, proteins were eluted with a linear NaCl gradient (0-0.5M). Alternate fractions were assayed for NOS activity using the oxy-hemoglobin assay. Total protein content was determined by Bradford's method.

Fig. 4. Fractionation of tobacco NOS activity by ARG-Sepharose chromatography. Fractions containing peak NOS activity (fractions 3-15) from DEAE-Sepharose chromatography were pooled and loaded onto an ARG-Sepharose column. The proteins were eluted with a linear NaCl gradient (0-0.5 M) and alternate fractions were assayed for NOS activity using arginine (Arg) and N-hydroxy-L-arginine (NOHA) as substrates. Total protein content was determined by Bradford's method.

Fig. 5. Protein profiles and NOS activity in various fractions eluted from ARG-Sepharose. Protein extracts from TMV-infected leaves were subjected to centrifugation, G-25 Sephadex, differential ammonium sulfate fractionation followed by DEAE-Sepharose chromatography and Arginine-Sepharose chromatography. Fractions (Fr.) were analyzed by SDS-PAGE (10%), followed by silver staining and by an oxy-hemoglobin assay. NOS activity is show above each gel track in pmol/min, using L-arginine (Arg) and N-hydroxy-L-arginine (NOHA) as substrates. Asterisk indicates the position of the polypeptide copurifying with NOS activity. Molecular weight markers are indicated along the side of the gel.

Fig. 6. Fractionation of tobacco NOS activity by ADP-Sepharose chromatography. Fractions containing peak NOS activity (Fractions 9-15) from ARG-Sepharose chromatography were pooled and loaded onto an ADP-Sepharose

column. The proteins were eluted with a linear NaCl gradient (0-0.5 M) and alternate fractions were assayed for NOS activity using L-arginine (Arg) and N-hyroxy-L-arginine (NOHA) as substrates. Total protein content was determined by Bradford's method.

Fig. 7. Protein profiles and NOS activity in various fractions eluted from ADP-Sepharose. Protein extracts from TMV-infected leaves were subjected to centrifugation, G-25 Sephadex, differential ammonium sulfate fractionation followed by DEAE-Sepharose chromatography, Arginine-Sepharose chromatography and ADP-Sepharose chromatography. Fractions (Fr.) were analyzed by SDS-PAGE (10%) followed by silver staining and by an oxy-hemoglobin assay. NOS activity is show above each gel track in pmol/min, using arginine (Arg) and N-hydroxy-L-arginine (NOHA) as substrates. Arrow indicates the position of the polypeptide copurifying with NOS activity. Molecular weight markers are indicated along the side of the gel.

Fig. 8. Fractionation of tobacco NOS activity by CaM-Sepharose chromatography. Fractions containing peak NOS activity (Fractions 3-9) from ADP-Sepharose chromatography were pooled and loaded onto a CaM-Sepharose column. The proteins were eluted with a linear gradient of EGTA (0-1.0 mM) and alternate fractions were assayed for NOS activity using L-arginine (Arg) and N-hyroxy-L-arginine (NOHA) as substrates. Total protein content was determined by Bradford's method.

Fig. 9. Protein profile and NOS-like activity in fractions 1-13 eluted from CaM affinity column. Crude extract prepared from TMV-infected Xanthi nc (NN)

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tobacco were subjected to differential ammonium sulfate precipitation, DEAE-sepharose ion exchange chromatography and arginine-sepharose and ADP-sepharose affinity chromatography. Fractions containing peak NOS-like activity from the ADP-sepharose fractionation step were pooled and loaded on a calmodulin (CaM)-sepharose column in presence of calcium chloride. The column was washed with low salt (50 mM NaCl) and proteins were eluted with 200 mM NaCl and 0-5 mM EGTA gradient. The protein profile of the various fractions is shown on a 10% SDS-polyacryamide gel with silver staining. The positions of molecular weight size markers in kDa are indicated at the left of the gel. NOS-like activity was assayed using either L-arginine (Arg) or N-hydroxy-L-arginine (NOHA) as substrate in the oxy-hemoglobin assay and is shown above each fraction in pmol/min/25 ul. The four major protein bands labeled a-d in fraction 5, which contains the highest level of NOS-like activity, were identified by mass spectroscopy (MALDI-TOF or Q-TOF). a and b ,which can not be really distinguished on the reproduction of the silver stained gel, were excised separately; a corresponds to a variant P subunit of glycine decarboxylase (GDC) while b is glutamate synthase (GltS). c is the large subunit (LSU) of Rubisco and d is plastidic aldolase. Note that LSU does not copurify with the NOS-like activity, while by silver staining there is no detectable aldolase in fractions 1, 11, and 13, yet there is detectable NOS-like activity. In contrast, the variant P subunit of GDC and GltS appear to copurify with the NOS-like activity.

Fig. 10. Comparison of NOS activity detected by oxy-hemoglobin assay and citrulline assay. NOS activity was measured in crude extract and pooled peak fractions of each step of the purification from tobacco, using the oxy-hemoglobin assay and the citrulline assay. Data shown are averages of three determinations. C,

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crude; DEAE, DEAE-Sepharose; ADP, ADP-Sepharose; ARG, Arginine-Sepharose; CaM, calmodulin-Sepharose.

Fig. 11. SDS-PAGE analysis of fractions from each steps of the NOS purification protocol. An aliquot of fractions containing peak NOS activity from each purification step was subjected to SDS-PAGE (10 %) and proteins were visualized by Coomassie blue (track 2-4) or silver (track 5-8) staining. Track 1-7 are from the same gel while track 8 is from the gel shown in Fig. 9 which corresponds to fraction number 5 from the CaM affinity column. The size of the molecular weight (M.W.) markers are given in kDa along the side of the gel. C, crude; G-25, G-25-Sephadex; (NH4)2SO4, ammonium sulfate fraction; DEAE, DEAE-Sepharose; ADP, ADP-Sepharose; ARG, Arginine-Sepharose; CaM, calmodulin-Sepharose; M, M.W. markers.

Fig. 12. Tryptophan fluorescence changes of tobacco NOS response to CaM binding. Fluorescence emission of NOS protein was recorded in the absence of calcium and CaM and in the presence of Ca^{2+} (100 μM) and different concentrations of CaM (μM) and EDTA (μM) as indicated in the inset. The protein was irradiated at 288-300 nm and emission spectra were recorded at 300-390 nm. As viewed from the trace at 340 nm, control (no additions) is third line from bottom; +Ca+CaM (0.1) is fourth line from bottom (and top); +Ca+CaM (0.5) is third line from top; +Ca+CaM (1.0) is second line from top; +Ca+CaM (3.0) is top line; +Ca+CaM (3.0) +EDTA (200) is second line from bottom; and +Ca+CaM (3.0) +EDTA (800) is bottom line.

Fig. 13. Flavin fluorescence changes of tobacco NOS in response to CaM binding. Fluorescence emission of NOS protein was recorded in the absence of

calcium and CaM and in the presence of Ca^{2+} (100 μM) and different concentrations of CaM (μM) and EDTA (μM) as indicated in the inset. The protein was irradiated at 450-460 nm and emission spectra were recorded at 500-650 nm. As viewed from the trace at about 530 nm, control (no additions) is second line from bottom; +Ca+CaM (0.1) is third line from bottom (and top); +Ca+CaM (0.5) is second line from top; +Ca+CaM (3.0) is top line; and +Ca+CaM (3.0) +EDTA (800) is bottom line.

Fig. 14. Effect of NOS inhibitors on activity of tobacco NOS. Effects of different concentrations of inhibitors L-NMMA and aminoguanidine were tested on purified tobacco NOS using the oxy-hemoglobin assay.

Fig. 15A. The deduced amino acid sequence of the *Arabidopsis* candidate P subunit (canP) of glycine decarboxylase. The sequence of the four peptides underlined correspond to those determined by mass spectroscopy (Q-TOF) of the putative tobacco NOS-like enzyme.

Fig. 15B. Critical motifs of the *Arabidopsis* glycine decarboxylase variant P subunit, including those shared with mammalian NOSs. Note that plant P subunits have a ~80 amino acid extension at their N-termini which is not present on animal or microbial P subunits. From left to right on the diagram, the first box on the bar is a CaM binding site; second box on the bar is a heme-oxygenase active site; third box on the bar is a FAD/FMN binding site; bar beneath third box on bar is NADPH-dependent cyt P450 reductase domain; fourth box on the bar is a leucine zipper; fifth and last box on the bar is a pyridoxal phosphate binding domain; short bar beneath fifth box is a potential heme attachment site; and longer bar beneath fifth box is a NADPH binding-Rossmann fold domain.

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Fig. 16. Comparison of NOS activity from tobacco in presence of tetrahydrobiopterin (H_4B) and tetrahydrofolate (THF). THF is a form of biopterin, known to function like H_4B to facilitate NO synthesis by some NOSs. The NOS activity was assessed by using a highly purified fraction of the tobacco enzyme in the presence of arginine (Arg) or N-hydroxy-L-arginine (NOHA) as substrate with the oxy-hemoglobin assay. The NOS assay reaction (NOSRx) contained 25 µl enzyme, Ca^{2+}, CaM, H_4B, FAD, NADPH, THF/H_4B and hemoglobin in 20 mM Hepes (pH 7.0).

Fig. 17. Effect of the P subunit inhibitor carboxy-methoxylamine on the NOS activity from tobacco. The effect of the inhibitor carboxymethoxylamine (CM) of P subunit of GDC on the highly purified tobacco NOS activity was assessed by using arginine (Arg) or N-hydroxy-L-arginine (NOHA) as substrate in the oxy-hemoglobin assay. Pyridoxal phosphate (PP) is known to substitute for the NADPH requirement in PP-dependent enzyme such as the P subunit of GDC. Thus, the effect of 200 µM CM was monitored in presence, as well as absence, of PP substituting for NADPH. The NOS assay reaction (NOSRx) contained 25 µl enzyme, Ca^{2+}, CaM, H_4B, FAD, (+/-)NADPH, (+/-)PP and hemoglobin in 20 mM Hepes (pH 7.0).

Fig. 18. Changes in NOS activity in *Arabidopsis* ecotype Col-0 and Di-17 after turnip crinkle virus (TCV) infection. Changes in NOS activity from 0 to 72 (hpi) with TCV in susceptible Col-0 and resistant Di-17 plants were measured by an oxy-hemoglobin assay.

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Fig. 19. Protein profiles of crude total extracts from canP transformed *E.coli* or soluble fraction before or after purification by Ni+, ARG or ADP affinity chromatography. Crude total extracts were prepared before or 6 hr after induction with 1 mM IPTG. The soluble fraction was prepared 6 hr after induction and subjected to affinity chromatography on Ni^+ column or sequential affinity chromatography on ARG-Sepharose and ADP-Sepharose. The NO synthesizing activity was determined with an oxy-hemoglobin assay using either L-arginine or N-hydroxy-L-arginine (NOHA) as substrate and supplemented with cofactors H_4B and heme. Activity is indicated below each track and expressed in pmol/mg/min. Total extract corresponds to Expt #1 in Table 3 while Ni^+ affinity purified fractions correspond to Expt #2 in Table 3. Asterisks mark the position of recombinant NOS/canP protein.

Fig. 20. Proposed SA- and NO- mediated pathway for activation of certain defense genes and elevation of intracellular free Fe^{2+}. Key signaling molecules in the cascade include NO, cGMP, cADPR, Ca^{2+}, and SA. Important enzymes are NOS, GC, cGMP-dependent protein kinase, and ADPRC. PAL and PR-1 are two important defense genes activated by pathogens. Activation of NOS by TMV infection increases NO levels, which activate GC and lead to elevated cGMP levels. cGMP activates ADPRC, via a cGMP-dependent protein kinase, which results in rising cADPR levels. CADPR activates ruthenium red (RR)-sensitive Ca^{2+} ion channels, which leads to higher cytosolic Ca^{2+} levels. Ca^{2+} induces SA biosynthesis, perhaps by activation of PAL gene expression. The SA-induced MAP kinase, SIPK, may play a role in SA signaling through NPR1. NPR1 transmits the SA signal to the PR-1 gene *via* its interaction with members of the TGA/OBF family of transcription

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factors, which bind the SA-responsive TGACG element of the PR-1 promoter. In a separate branch of the pathway, NO inhibits cytosolic aconitase and may convert it into an iron regulatory protein (IRP) thereby facilitating an increase in intracellular free Fe^{2+}. NO also inhibits catalase and ascorbate peroxidase, the two major H_2O_2-scavanging enzymes. Free Fe^{2+} catalyzes the conversion of H_2O_2 to the extremely reactive OH:. GSNO and SNAP are NO donors, whereas PTIO is an NO scavenger.

DETAILED DESCRIPTION OF THE INVENTION

Various terms relating to the biological molecules and other aspects of the present invention are used throughout the specification and claims.

"NOS", "piNOS" or "NOS-like enzyme" refers generally to a polypeptide in accordance with the present invention, which is described in detail herein above and throughout the specification.

"NOS activity" or "NOS-like activity" or "biological activity of the NOS-like enzyme" refers to the metabolic or physiologic function of the plant NOS of the invention, including similar activities or improved activities or these activities with decreased undesirable side-effects. Also included are antigenic and immunogenic activities of the plant NOS of the invention.

"Isolated" means altered "by the hand of man" from the natural state. If a composition or substance occurs in nature, it has been "isolated" if it has been changed or removed from its original environment, or both. For example, a polynucleotide or a polypeptide naturally present in a living plant or animal is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein.

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"Polynucleotide", also referred to as "nucleic acid molecule", generally refers to any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. "Polynucleotides" include, without limitation single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, "polynucleotide" refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term polynucleotide also includes DNAs or RNAs containing one or more modified bases and DNAs or RNAs with backbones modified for stability or for other reasons. "Modified" bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications can be made to DNA and RNA; thus, "polynucleotide" embraces chemically, enzymatically or metabolically modified forms of polynucleotides as typically found in nature, as well as the chemical forms of DNA and RNA characteristic of viruses and cells. "Polynucleotide" also embraces relatively short polynucleotides, often referred to as oligonucleotides.

"Polypeptide" refers to any peptide or protein comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds, i.e., peptide isosteres. "Polypeptide" refers to both short chains, commonly referred to as peptides, oligopeptides or oligomers, and to longer chains, generally referred to as proteins. Polypeptides may contain amino acids other than the 20 gene-encoded amino acids. "Polypeptides" include amino acid sequences modified either by natural processes, such as posttranslational processing, or by chemical modification techniques which

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are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. Polypeptides may be branched as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched and branched cyclic polypeptides may result from natural posttranslational processes or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. See, for instance, Proteins - Structure and Molecular Properties, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York, 1993 and Wold, F., Posttranslational Protein Modifications: Perspectives and Prospects, pgs. 1-12 in Posttranslational Covalent Modification of Proteins, B. C. Johnson, Ed., Academic Press, New York, 1983; Seifter et al., "Analysis for Protein Modifications and

Nonprotein Cofactors", Meth Enzymol (1990) 182:626-646 and Rattan et al., "Protein Synthesis: Posttranslational Modifications and Aging", Ann NY Acad Sci (1992) 663:48-62.

"Variant" as the term is used herein, is a polynucleotide or polypeptide that differs from a reference polynucleotide or polypeptide respectively, but retains essential properties. A typical variant of a polynucleotide differs in nucleotide sequence from another, reference polynucleotide. Changes in the nucleotide sequence of the variant may or may not alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Nucleotide changes may result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence, as discussed below. A typical variant of a polypeptide differs in amino acid sequence from another, reference polypeptide. Generally, differences are limited so that the sequences of the reference polypeptide and the variant are closely similar overall and, in many regions, identical. A variant and reference polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions in any combination. A substituted or inserted amino acid residue may or may not be one encoded by the genetic code. A variant of a polynucleotide or polypeptide may be naturally occurring, such as an allelic variant, or it may be a variant that is not known to occur naturally. Non-naturally occurring variants of polynucleotides and polypeptides may be made by mutagenesis techniques or by direct synthesis.

In reference to mutant plants, the terms "null mutant" or "loss-of-function mutant" are used to designate an organism or genomic DNA sequence with a mutation that causes the product of the piNOS-encoding gene to be non-functional or

largely absent. Such mutations may occur in the coding and/or regulatory regions of the gene, and may be changes of individual residues, or insertions or deletions of regions of nucleic acids. These mutations may also occur in the coding and/or regulatory regions of other genes which may regulate or control the piNOS-encoding gene and/or the piNOS enzyme, so as to cause the enzyme to be non-functional or largely absent.

The term "disease defense response" refers to a change in metabolism, biosynthetic activity or gene expression that enhances the plant's ability to suppress the replication and spread of a microbial pathogen (i.e., to resist the microbial pathogen). Examples of plant disease defense responses include, but are not limited to, production of low molecular weight compounds with antimicrobial activity (referred to as phytoalexins) and induction of expression of defense (or defense-related) genes, whose products include, for example, peroxidases, cell wall proteins, proteinase inhibitors, hydrolytic enzymes, pathogenesis-related (PR) proteins and phytoalexin biosynthetic enzymes, such as phenylalanine ammonia lyase and chalcone synthase. Such defense responses appear to be induced in plants by several signal transduction pathways involving secondary defense signaling molecules produced in plants. Agents that induce disease defense responses in plants (which are also referred to herein as "disease-causing agents"), include but are not limited to: (1) microbial pathogens, such as fungi, bacteria and viruses; (2) microbial components and other defense response elicitors, such as proteins and protein fragments, small peptides, β-glucans, elicitins and harpins, cryptogein and oligosaccharides; and (3) secondary defense signaling molecules produced by the plant, such as NO, SA, H_2O_2, ethylene and jasmonates.

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The terms "defense-related genes" and "defense-related proteins" refer to genes or their encoded proteins whose expression or synthesis is associated with (induced after) infection with a pathogen to which the plant is usually resistant.

The term "substantially the same" refers to nucleic acid or amino acid sequences having sequence variations that do not materially affect the nature of the protein (i.e. the structure, stability characteristics, substrate specificity and/or biological activity of the protein). With particular reference to nucleic acid sequences, the term "substantially the same" is intended to refer to the coding region and to conserved sequences governing expression, and refers primarily to degenerate codons encoding the same amino acid, or alternate codons encoding conservative substitute amino acids in the encoded polypeptide. With reference to amino acid sequences, the term "substantially the same" refers generally to conservative substitutions and/or variations in regions of the polypeptide not involved in determination of structure or function.

The terms "percent identical" and "percent similar" are also used herein in comparisons among amino acid and nucleic acid sequences. When referring to amino acid sequences, "identity" or "percent identical" refers to the percent of the amino acids of the subject amino acid sequence that have been matched to identical amino acids in the compared amino acid sequence by a sequence analysis program. "Percent similar" refers to the percent of the amino acids of the subject amino acid sequence that have been matched to identical or conserved amino acids. Conserved amino acids are those which differ in structure but are similar in physical properties such that the exchange of one for another would not appreciably change the tertiary structure of the resulting protein. Conservative substitutions are defined in Taylor (1986, J.

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Theor. Biol. 119:205). When referring to nucleic acid molecules, "percent identical" refers to the percent of the nucleotides of the subject nucleic acid sequence that have been matched to identical nucleotides by a sequence analysis program.

"Identity" and "similarity" can be readily calculated by known methods. Nucleic acid sequences and amino acid sequences can be compared using computer programs that align the similar sequences of the nucleic or amino acids and thus define the differences. In preferred methodologies, the BLAST programs (NCBI) and parameters used therein are employed, and the DNAstar system (Madison, WI) is used to align sequence fragments of genomic DNA sequences. However, equivalent alignments and similarity/identity assessments can be obtained through the use of any standard alignment software. For instance, the GCG Wisconsin Package version 9.1, available from the Genetics Computer Group in Madison, Wisconsin, and the default parameters used (gap creation penalty=12, gap extension penalty=4) by that program may also be used to compare sequence identity and similarity.

"Antibodies" as used herein includes polyclonal and monoclonal antibodies, chimeric, single chain, and humanized antibodies, as well as Fab fragments, including the products of an Fab or other immunoglobulin expression library. With respect to antibodies, the term, "immunologically specific" refers to antibodies that bind to one or more epitopes of a protein of interest, but which do not substantially recognize and bind other molecules in a sample containing a mixed population of antigenic biological molecules.

The term "substantially pure" refers to a preparation comprising at least 50-60% by weight the compound of interest (e.g., nucleic acid, oligonucleotide, protein, etc.). More preferably, the preparation comprises at least 75% by weight, and most

preferably 90-99% by weight, the compound of interest. Purity is measured by methods appropriate for the compound of interest (e.g. chromatographic methods, agarose or polyacrylamide gel electrophoresis, HPLC analysis, and the like).

With respect to single-stranded nucleic acid molecules, the term "specifically hybridizing" refers to the association between two single-stranded nucleic acid molecules of sufficiently complementary sequence to permit such hybridization under pre-determined conditions generally used in the art (sometimes termed "substantially complementary"). In particular, the term refers to hybridization of an oligonucleotide with a substantially complementary sequence contained within a single-stranded DNA or RNA molecule, to the substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence.

A "coding sequence" or "coding region" refers to a nucleic acid molecule having sequence information necessary to produce a gene product, when the sequence is expressed.

The term "operably linked" or "operably inserted" means that the regulatory sequences necessary for expression of the coding sequence are placed in a nucleic acid molecule in the appropriate positions relative to the coding sequence so as to enable expression of the coding sequence. This same definition is sometimes applied to the arrangement other transcription control elements (e.g. enhancers) in an expression vector.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell.

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The terms "promoter", "promoter region" or "promoter sequence" refer generally to transcriptional regulatory regions of a gene, which may be found at the 5' or 3' side of the coding region, or within the coding region, or within introns. Typically, a promoter is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. The typical 5' promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence is a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

A "vector" is a replicon, such as plasmid, phage, cosmid, or virus to which another nucleic acid segment may be operably inserted so as to bring about the replication or expression of the segment.

The term "nucleic acid construct" or "DNA construct" is sometimes used to refer to a coding sequence or sequences operably linked to appropriate regulatory sequences and inserted into a vector for transforming a cell. This term may be used interchangeably with the term "transforming DNA" or "transgene". Such a nucleic acid construct may contain a coding sequence for a gene product of interest, along with a selectable marker gene and/or a reporter gene.

The term "selectable marker gene" refers to a gene encoding a product that, when expressed, confers a selectable phenotype such as antibiotic resistance on a transformed cell.

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The term "reporter gene" refers to a gene that encodes a product which is easily detectable by standard methods, either directly or indirectly.

A "heterologous" region of a nucleic acid construct is an identifiable segment (or segments) of the nucleic acid molecule within a larger molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. In another example, a heterologous region is a construct where the coding sequence itself is not found in nature (e.g., a cDNA where the genomic coding sequence contains introns, or synthetic sequences having codons different than the native gene). Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein. The term "DNA construct", as defined above, is also used to refer to a heterologous region, particularly one constructed for use in transformation of a cell.

A cell has been "transformed" or "transfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a

single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

NO is a key component in the signal transduction pathway(s) leading to activation of certain defense responses in plants after pathogen attack. Fig. 20 schematically depicts the central role postulated for NO in plant disease defense responses. In spite of the key role NO appears to occupy in plant disease defense signal transduction, it has remained undetermined how nitric oxide (NO) is produced in plants following an attack by microbial pathogens. Several studies, including those of the inventors, suggest that a nitric oxide synthase (NOS)-like enzyme is responsible, despite the absence of an obvious ortholog of animal NOSs in plants, including *Arabidopsis thaliana*, whose complete genome sequence is available.

In accordance with the present invention, a plant NOS-like enzyme (piNOS) has now been obtained. The enzyme was purified ~33,000 fold from TMV-infected tobacco via five fractionation steps, including three affinity chromatography columns. The purification of this enzyme is described in detail in Example 1. Characterization of the enzyme is described in Example 2.

The inventors have previously demonstrated that a NOS activity increases 4-6 fold in TMV-infected resistant tobacco plants. However, the actual level of NOS activity in crude extracts prepared as described by Durner et al. (1998, Proc. Natl. Acad. Sci. USA 95: 10328-10333) was highly variable. In order to purify the tobacco NOS, the inventors therefore modified the extraction and assay conditions and employed an oxy-hemoglobin assay, in addition to the citrulline assay, for NOS activity (Kelm, M., et al., 1988, Biochem. Biophys. Res. Commun. 154: 236-244.;

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Baek, K.J., et al.,1993, J. Biol. Chem. 268: 21120-21129; Murphy, M.E. and Noack, E.,1994, Methods Enzymol. 233: 240-250.; Durner et al.,1998, *supra*). The oxy-hemoglobin assay is based on a direct reaction between NO and the oxygenated, ferrous form of hemoglobin (HbO_2), which yields the ferric form, methemoglobin (metHb), and nitrate. The rate of NO production arising from NOS activity is quantified by measuring the conversion of HbO_2 to metHb. To ensure that metHb production accurately measured NO synthesis in our assay, metHb production also was monitored in the presence of nitro-L-arginine. This arginine analog inhibits NO synthesis; therefore any metHb production in its presence is due to non-specific HbO_2 oxidation and was subtracted from the level of metHb produced in the absence of this inhibitor.

With the optimized extraction and assay conditions, induction of the NOS activity in resistant tobacco was found to be very rapid and much more dramatic than previously reported (Fig. 1A and B). TMV-inoculated plants from the resistant tobacco cultivar Xanthi nc (NN) exhibited a 10-18 fold increase in NOS activity following a temperature shift. By contrast, NOS activity remained unchanged in plants from the susceptible cultivar Xanthi (nn) (Fig. 1A). An even greater increase in NOS activity (25-30 fold) was observed in TMV-inoculated Xanthi nc (NN) plants maintained at 22°C; a 5 fold increase was evident as early as 2 hours after infection (Fig. 1B). NOS activity was dependent on the substrate arginine and the cofactors NADPH, FMN, FAD and CaM. Strikingly, infection with the non-host pathogen *Pseudomonas syringe* pv. *maculicola*, which induces an HR and other defenses, also led to a large (~7 fold), although transient, rise in NOS activity (Fig. 2). Therefore,

increases in a NOS-like activity are associated with activation of defense responses by two avirulent pathogens of tobacco.

To ensure accurate monitoring of NOS activity throughout the purification process, a citrulline assay, which is the standard assay used in animal systems, was employed (Durner, J., et al., 1997, Trends Plant Sci. 2: 266-274) in addition to the oxy-hemoglobin assay. Both the citrulline assay, which measures the formation of ^{3}H-citrulline from ^{3}H-L-arginine, and the oxy-hemoglobin assay gave similar results with the crude extract and fractions from various purification steps. For additional confirmation, oxy-hemoglobin assays were performed in parallel, using N-hydroxy-L-arginine (NOHA), as well as arginine, for the substrate. NOHA is the intermediate in the conversion of arginine to NO and citrulline by all eukaryotic NOSs; both arginine and NOHA gave similar results.

To further establish that the tobacco enzyme is a NOS-like enzyme, its requirement for CaM was assessed. CaM binding to animal NOS increases its activity by enhancing the rate of electron transfer from NADPH to the flavins, and from the flavins to the heme, which results in increased NO synthesis. CaM-mediated activation of the reductase domain can be monitored by measuring changes in tryptophan and flavin fluorescence (Gachhui, R., et al., 1996, J. Biol. Chem. 271: 20594-20602). As anticipated, the activity of the purified tobacco enzyme was enhanced by CaM. Additionally, the presence of Ca^{2+} and increasing concentrations of CaM enhanced the level of tryptophan and flavin fluorescence. This increase in fluorescence could be reversed by EDTA, which chelates Ca^{2+} and causes CaM to dissociate from the NOS.

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Additional evidence that the purified tobacco enzyme is NOS-like came from inhibitor studies. Two of the most widely used inhibitors of animal NOSs are N(G)-monomethyl-L-arginine (L-NMMA) and aminoguanidine. Both inhibitors suppressed the activity of the purified tobacco enzyme in a concentration dependent manner, with L-NMMA providing the highest level of inhibition.

In summary, the biochemical characteristics of the purified tobacco enzyme are highly similar to those of animal NOSs (see Example 2 and figures and tables referred to therein). Therefore, the inventors concluded that this protein is a NOS whose DNA and amino acid (aa) sequences have sufficiently diverged from those of animals, making it unidentifiable using molecular or bioinformatic approaches.

Rigorous proof that the TMV-induced NOS-like activity was the product of a plant NOS-like enzyme was provided by (i) purification of the activity 33,000-fold (see Table 1 in Example 1), (ii) partial determination of the amino acid sequence of the protein that copurified with the NOS-like activity (Fig. 15A), (iii) identification in the deduced protein sequence of the corresponding gene in *Arabidopsis thaliana* of the critical motifs required for NOS activity and shared with mammalian NOSs (Fig. 15B) and (iv) the demonstration that the products of the corresponding *Arabidopsis* gene, produced in *E. coli* or recombinant baculovirus-infected cells, possessed NOS activity.

The four most prevalent proteins present in the most highly purified tobacco fraction described in Example 1 and shown in Fig. 9 were fragmented with trypsin and subjected to mass spectroscopy. This analysis revealed that the ~120 kDa protein (labeled "a" in Fig. 9), which was found to copurify with NOS-like activity in several independent experiments, is a variant of the P subunit of glycine decarboxylase

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(GDC). The ~115 kDa protein is ferredoxin-dependent glutamate synthase (Fd-GltS) and the two lower molecular weight proteins are the large subunit of RuBisCO and plastitic aldolase, respectively. Mass spectroscopic analysis of other preparations of highly purified NOS-like activity similarly indicated that the high molecular weight band copurifying with the NOS-like activity is the P subunit of GDC. In *Arabidopsis*, two genes encode the P subunit of GDC. Since the deduced amino acid sequence of only one of these *Arabidopsis* genes exactly matched that of the four tryptic peptides obtained from the tobacco NOS-like enzyme, it is expected that this protein has NOS activity in *Arabadopsis*. Accordingly, the corresponding *Arabidopsis* gene was designated *CanP* (for the P subunit that is a candidate for NO synthesizing activity) and its deduced amino acid sequence is shown in Fig. 15A. By contrast, the deduced amino acid sequence of the second *Arabidopsis* P subunit gene, referred to as P2, exactly matches only two of the four peptides from the tobacco NOS-like enzyme, but differs from the remaining peptides at several residues. Structural analysis of *CanP* indicated that it contains most, if not all, of the motifs necessary for synthesis of NO from arginine (Fig. 15B). These motifs are similar in sequence (e.g. cofactor-binding sites) and/or function to those found in mammalian NOSs and include, from the N- to C-terminus: (1) a CaM-binding site (2) a heme oxygenase active site; (3) an FAD/FMN-binding site; (4) an NADPH-dependent cytochrome P_{450} reductase domain; (5) a potential heme attachment site; and (6) an NADPH-binding –Rossmann fold domain that also binds tetrahydrofolate. This latter observation is notable, since NOS from the bacterium *Deinococcus radiodurans* can use THF in place of tetrahydrobiopterin (H_4B), the cofactor utilized by mammalian NOSs. In fact, the tobacco NOS-like enzyme has a slight preference for THF over H_4B (Fig. 16).

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The *Arabidopsis* P2 protein shares many structural motifs with *CanP*, and both are very similar in sequence (overall identity 89% and similarity 92%) except for their N-termini, which share only 51 of 80 residues in common. However, P2 is missing the heme oxygenase active site (residues 32-44), which is functionally similar to the mammalian NOS oxygenase domain, and therefore is unlikely to have NO synthesizing activity. It should be noted in this regard that the plant P subunits of GDC have an N-terminal extension of about 80 amino acids that is not found on animal or microbial P subunits. Since many of the critical motifs for NOS activity are in this N-terminal extension, the animal and microbial P subunits are very unlikely to have NOS activity. All of the plant P subunits sequenced thus far have N-terminal extensions and one or more of these critical motifs; however, none have all the prerequisite motifs, arguing that they also do not have NOS activity. Perhaps this finding is not surprising, since these genes likely encode the major P subunit isoform, which is expressed at high levels in green tissue and is part of the multi-protein/subunit (P, H, T and L) GDC complex. The GDC complex, which represents 25-40% of the protein in mitochondria, converts glycine, produced indirectly as a result of the oxygenase activity of RuBisCO (photorespiration), into CO_2, NH_3 and, with the help of serine hydroxymethyltransferase, serine. By contrast, orthologs of the tobacco and *Arabidopsis CanP* gene probably are expressed at low levels and/or only under certain conditions, such as during a resistance response, given the extremely low level of NOS-like activity detected even in tobacco resisting TMV infection.

In addition to copurifying with NOS activity and containing all of the prerequisite motifs required for NO synthesis, two other lines of evidence strongly

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indicate that *CanP* and its tobacco ortholog are NOS-like enzymes. Inhibitors that specifically block the activity of the P subunit, such as carboxymethoxylamine and aminoacetonitrile, also were found to inhibit NOS-like activity in both a relatively crude preparation of the tobacco enzyme (before affinity chromatography; data not shown) and the highly purified enzyme (Fig. 17). Moreover, expression of recombinant His6-tagged *CanP* in *E. coli* resulted in a 17-62 fold increase in NO synthesizing activity using either L-arginine or NOHA as substrates. This increase corresponded with the accumulation of a high molecular weight protein of ~120 kDa that was greatly enriched by chromatography on a Ni column (Fig. 19). Similarly, "Sf9" insect cells infected with recombinant baculovirus expressing His6-tagged *CanP* exhibited a 7-32 fold increase in NOS-like activity compared with mock- or empty baculovirus vector- infected cells.

Although the piNOS purified from tobacco and the piNOS produced recombinantly from an *Arabidopsis* cDNA are described and exemplified herein, this invention is intended to encompass proteins from other species that are sufficiently similar to be used interchangeably with tobacco or *Arabidopsis* NOSs for the purposes described below. Accordingly, when the terms "piNOS", "NOS" or "NOS-like enzyme" are used herein, they are intended to encompass all plant NOSs having the general physical, biochemical and functional features described herein. Tobacco and *Arabidopsis* NOSs are exemplary members of this group, and the group includes homologs of the tobacco or *Arabidopsis* enzyme isolated from any plant species. This group does not include nitrate reductase, which is capable of producing NO *via* a secondary reaction from nitrite, but does not produce NO from arginine, the substrate for NOSs, including piNOS.

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Considered in terms of their sequences, piNOSs and *piNOS* genes of the invention include allelic variants and natural mutants of SEQ ID NO:6, which are likely to be found in different varieties of *Arabidopsis*, and homologs of SEQ ID NO:6 likely to be found in different plant species. Because such variants and homologs are expected to possess certain differences in nucleotide and amino acid sequence, this invention provides an isolated piNOS-encoding nucleic acid molecule that encodes a piNOS polypeptide having at least about 90% (and, with increasing order of preference, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% and 99%) identity with SEQ ID NO:1, and comprises a nucleotide sequence having equivalent ranges of identity to SEQ ID NO:6. The 80-residue amino terminal portion of piNOS comprises most of the NOS functional motifs and differs much more than other segments of the protein from the protein that forms part of the GDC complex. Accordingly, preferred embodiments of the invention are drawn to piNOSs and piNOS-encoding nucleic acids wherein the 80-or-so residue amino terminus of the encoded protein comprises at least 60% (and, with increasing order of preference, 65%, 70%, 75%, 80%, 85%, 90% and 95%) sequence identity in the coding regions with the corresponding region of SEQ ID NO:1, and comprises a nucleotide sequence having equivalent ranges of identity to SEQ ID NO:6. Because of the natural sequence variation likely to exist among pNOS enzymes and the genes encoding them in different plant varieties and species, one skilled in the art would expect to find this level of variation, while still maintaining the unique properties of the piNOS of the present invention. Such an expectation is due in part to the degeneracy of the genetic code, as well as to the known evolutionary success of conservative amino acid sequence variations, which do not appreciably alter the nature of the encoded protein.

Accordingly, such variants and homologs are considered substantially the same as one another and are included within the scope of the present invention.

The following sections set forth the general procedures involved in practicing the present invention. To the extent that specific materials are mentioned, it is merely for the purpose of illustration, and is not intended to limit the invention. Unless otherwise specified, general biochemical and molecular biological procedures, such as those set forth in Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory (1989) or Ausubel et al. (eds), Current Protocols in Molecular Biology, John Wiley & Sons (2002) are used.

A piNOS protein of the present invention may be prepared in a variety of ways, according to known methods. The protein may be purified from appropriate sources, e.g., plant cells or tissues as described in detail in Example 1. That example describes the isolation of piNOS from TMV-inoculated tobacco, followed by its 33,000-fold purification.

The availability of sufficient quantities of substantially pure piNOS from tobacco, prepared as described herein, enables the amino acid sequence of the protein to be determined, in accordance with standard methods. Briefly, the highly purified piNOS can be fractionated on a 7.5% SDS-polyacrylamide gel and the activity-containing band excised after staining with Coomassie Brilliant Blue R250/silver nitrate. The excised protein is subjected to digestion with trypsin, followed by amino acid sequence analysis. Tryptic fragments of the tobacco piNOS of the invention possess the following sequences, which are also present in the deduced amino acid sequence of the piNOS from *Arabidopsis*, (SEQ ID NO:1) as shown in Fig. 15A.

FGVPMGYGGPHAAFLATSQEYKR (SEQ ID NO:2)

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GNINIEEVRK (SEQ ID NO:3)

IAILNANYMAK (SEQ ID NO:4)

GNADVQNNVL (SEQ ID NO:5)

The availability of amino acid sequence information for the plant NOSs further enables cloning of cDNAs or genes encoding the proteins. This is also accomplished according to methods well known in the art. For example, the sequences of two piNOS peptides with low degeneracy in code may be used to design sets of forward and backward oligonucleotide primers. Specifically, two combinations of forward plus backward primers may be used to PCR amplify a segment of the gene from cDNA prepared from an expression library, for instance, a Zap library generated with poly(A) RNA from TMV-infected tobacco leaves (see, e.g., Guo, A., et al., 1998, Plant J. 15: 647-656). The resultant PCR-generated DNA fragment is sequenced and used as a probe to obtain a cDNA clone from the expression library. Cloning and amplification of a cDNA encoding a NOS from *Arabidopsis* (SEQ ID NO: 6) is described in Example 3.

Once a cDNA clone encoding a polypeptide with characteristics of piNOSs is obtained, a full-length cDNA clone may be obtained, if necessary, using the 5' RACE methodology, then sequenced. To confirm that it encodes NOS, the cDNA may be expressed in *E. coli*, yeast or baculovirus-infected insect cells, as is well known in the art. The baculovirus-Sf9 cell system is particularly suitable, having been developed for optimum production of recombinant proteins. The recombinant enzyme, synthesized as a his6 or GST-tagged (e.g., vector pET-28a or pGEX-5X-2, respectively) protein, is partially purified using a nickel column or glutathione

sepharose column, respectively and tested for NOS activity. Expression of an *Arabidopsis* NOS-encoding cDNA (SEQ ID NO:6) in *E. coli* and baculovirus-infected insect cells is described in Example 3.

Plant NOS purified from tobacco or other plant sources as described above or, in the alternative, recombinantly produced piNOS, may be used to generate polyclonal or monoclonal antibodies (Ab), according to known methods. In addition to making Ab to the entire recombinant protein, if analyses of the purified proteins (see above) or Southern and cloning analyses (see below) indicate that the cloned NOS belongs to a multigene family, then member-specific Ab made to synthetic peptides corresponding to nonconserved regions of the protein can be generated. In the present invention, antibodies specific to the amino terminus of the piNOS enzyme (approximately 80 residues) are expected to be of particular utility due to their ability to distinguish piNOS from similar proteins that form part of the plant's GDC complex, but lack most of the NOS functional motifs.

Upon obtaining sequence information from a plant NOS gene, cDNA, or corresponding protein, e.g., from tobacco, this sequence is useful for searching for NOS orthologs in other plant species, as the inventors have done in *Arabidopsis*. *Arabidopsis* is an ideal plant to analyze disease resistance because its entire genome has been sequenced and an extensive collection of mutants exhibiting altered disease resistance and/or SA-mediated defense signaling has been generated. The candidate NOS gene(s) may be expressed in one of the above expression systems and its purified recombinant protein tested for NOS activity, as described in Example 3.

In accordance with the present invention, nucleic acids having the appropriate level sequence homology with part or all of the coding and/or regulatory regions of

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piNOS-encoding polynucleotides may be identified by using hybridization and washing conditions of appropriate stringency. Since the piNOS is similar in sequence to the P subunit of GDC except in the N-terminal 80 residues, a preferred nucleic acid segment for hybridization is the 5' end of the gene encoding these N-terminal residues.

As a typical illustration, hybridizations may be performed, according to the method of Sambrook et al., using a hybridization solution comprising: 5X SSC, 5X Denhardt's reagent, 1.0% SDS, 100 μg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide. Hybridization is carried out at 37-42°C for at least six hours. Following hybridization, filters are washed as follows: (1) 5 minutes at room temperature in 2X SSC and 1% SDS; (2) 15 minutes at room temperature in 2X SSC and 0.1% SDS; (3) 30 minutes-1 hour at 37°C in 2X SSC and 0.1% SDS; (4) 2 hours at 45-55°C in 2X SSC and 0.1% SDS, changing the solution every 30 minutes.

One common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules of a specified sequence homology (Sambrook et al., 1989):

$T_m = 81.5°C + 16.6\text{Log}\,[Na+] + 0.41(\%\ G+C) - 0.63\ (\%\ \text{formamide}) - 600/\#\text{bp in duplex}$

As an illustration of the above formula, using [Na+] = [0.368] and 50% formamide, with GC content of 42% and an average probe size of 200 bases, the Tm is 57°C. The T_m of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in

homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of 42°C. In one embodiment, the hybridization is at 37°C and the final wash is at 42°C; in another embodiment the hybridization is at 42°C and the final wash is at 50°C; and in yet another embodiment the hybridization is at 42°C and final wash is at 65°C, with the above hybridization and wash solutions. Conditions of high stringency include hybridization at 42°C in the above hybridization solution and a final wash at 65°C in 0.1X SSC and 0.1% SDS for 10 minutes.

Nucleic acids of the present invention may be maintained as DNA in any convenient cloning vector. In a preferred embodiment, clones are maintained in plasmid cloning/expression vector, such as pGEM-T (Promega Biotech, Madison, WI) or pBluescript (Stratagene, La Jolla, CA), either of which is propagated in a suitable *E. coli* host cell.

piNOS-encoding nucleic acid molecules of the invention include cDNA, genomic DNA, RNA, and fragments thereof which may be single- or double-stranded. Thus, this invention provides oligonucleotides (sense or antisense strands of DNA or RNA) having sequences capable of hybridizing with at least one sequence of a nucleic acid molecule of the present invention. Such oligonucleotides are useful as probes for detecting piNOS-encoding genes or mRNA in test samples of plant tissue, e.g. by PCR amplification, or for the positive or negative regulation of expression of piNOS-encoding genes at or before translation of the mRNA into proteins. Methods in which piNOS-encoding oligonucleotides or polynucleotides may be utilized as probes for such assays include, but are not limited to: (1) *in situ* hybridization; (2) Southern

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hybridization (3) northern hybridization; and (4) assorted amplification reactions such as polymerase chain reactions (PCR) and ligase chain reaction (LCR).

Also featured in accordance with the present invention are vectors and kits for producing transgenic host cells that produce a piNOS enzyme. Suitable host cells include, but are not limited to, plant cells, bacterial cells, yeast and other fungal cells, insect cells and mammalian cells. Vectors for transforming a wide variety of these host cells are well known to those of skill in the art. They include, but are not limited to, plasmids, cosmids, baculoviruses, bacmids, bacterial artificial chromosomes (BACs), yeast artificial chromosomes (YACs), as well as other bacterial, yeast and viral vectors. Typically, kits for producing transgenic host cells that produce a piNOS enzyme will contain one or more appropriate vectors and instructions for producing the transgenic cells using the vector. Kits may further include one or more additional components, such as culture media for culturing the cells, reagents for performing transformation of the cells, reagents for testing the transgenic cells for piNOS-encoding gene expression and reagents for testing the transgenic cells for piNOS enzymatic activity, to name a few.

The present invention includes transgenic plants comprising one or more copies of a piNOS-encoding gene, or nucleic acid sequences that inhibit the production or function of a plant's endogenous piNOS. This is accomplished by transforming plant cells with a transgene that comprises part of all of a piNOS coding sequence, or mutant, antisense or variant thereof, including RNA, controlled by either native or recombinant regulatory sequences, as described below. Transgenic plants of any species are included in the invention; these include, but are not limited to, tobacco and *Arabidopsis*, cereal crops such as maize, wheat, rice, soybean barley, rye, oats,

sorghum, alfalfa, clover and the like, oil-producing plants such as canola, safflower, sunflower, peanut and the like, vegetable crops such as tomato tomatillo, potato, pepper, eggplant, sugar beet, carrot, cucumber, lettuce, pea and the like, horticultural plants such as aster, begonia, chrysanthemum, delphinium, zinnia, lawn and turfgrasses and the like.

Transgenic plants can be generated using standard plant transformation methods known to those skilled in the art. These include, but are not limited to, *Agrobacterium* vectors, polyethylene glycol treatment of protoplasts, biolistic DNA delivery, UV laser microbeam, gemini virus vectors or other plant viral vectors, calcium phosphate treatment of protoplasts, electroporation of isolated protoplasts, agitation of cell suspensions in solution with microbeads coated with the transforming DNA, agitation of cell suspension in solution with silicon fibers coated with transforming DNA, direct DNA uptake, liposome-mediated DNA uptake, and the like. Such methods have been published in the art. See, e.g., Methods for Plant Molecular Biology (Weissbach & Weissbach, eds., 1988); Methods in Plant Molecular Biology (Schuler & Zielinski, eds., 1989); Plant Molecular Biology Manual (Gelvin, Schilperoort, Verma, eds., 1993); and Methods in Plant Molecular Biology - A Laboratory Manual (Maliga, Klessig, Cashmore, Gruissem & Varner, eds., 1994).

The method of transformation depends upon the plant to be transformed. *Agrobacterium* vectors are often used to transform dicot species. *Agrobacterium* binary vectors include, but are not limited to, BIN19 and derivatives thereof, the pBI vector series, and binary vectors pGA482 and pGA492 . For transformation of monocot species, biolistic bombardment with particles coated with transforming DNA and silicon fibers coated with transforming DNA are often useful for nuclear

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transformation. Alternatively, *Agrobacterium* "superbinary" vectors have been used successfully for the transformation of rice, maize and various other monocot species.

DNA constructs for transforming a selected plant comprise a coding sequence of interest operably linked to appropriate 5' (e.g., promoters and translational regulatory sequences) and 3' regulatory sequences (e.g., terminators). In a preferred embodiment, a piNOS coding sequence under control of its own 5' and 3' regulatory elements is utilized.

In an alternative embodiment, the coding region of the gene is placed under a powerful constitutive promoter, such as the Cauliflower Mosaic Virus (CaMV) 35S promoter or the figwort mosaic virus 35S promoter. Other constitutive promoters contemplated for use in the present invention include, but are not limited to: T-DNA mannopine synthetase, nopaline synthase and octopine synthase promoters. In other embodiments, a strong monocot promoter is used, for example, the maize ubiquitin promoter, the rice actin promoter or the rice tubulin promoter (Jeon et al., Plant Physiology. 123: 1005-14, 2000).

Transgenic plants expressing piNOS coding sequences under an inducible promoter are also contemplated to be within the scope of the present invention. Inducible plant promoters include the tetracycline repressor/operator controlled promoter, the heat shock gene promoters, stress (e.g., wounding)-induced promoters, defense responsive gene promoters (e.g. phenylalanine ammonia lyase genes), wound induced gene promoters (e.g. hydroxyproline rich cell wall protein genes), chemically-inducible gene promoters (e.g., nitrate reductase genes, glucanase genes, chitinase genes, etc.) and dark-inducible gene promoters (e.g., asparagine synthetase

gene) to name a few. The use of pathogen- and wound-inducible promoters is described in more detail below.

Tissue specific and development-specific promoters are also contemplated for use in the present invention. Examples of these include, but are not limited to: the ribulose bisphosphate carboxylase (RuBisCo) small subunit gene promoters or chlorophyll a/b binding protein (CAB) gene promoters for expression in photosynthetic tissue; the various seed storage protein gene promoters for expression in seeds; and the root-specific glutamine synthetase gene promoters where expression in roots is desired.

In another embodiment, a piNOS coding region is operably linked to a heterologous promoter that is either generally pathogen inducible (i.e. inducible upon challenge by a broad range of pathogens) or wound inducible. Such promoters include, but are not limited to:

a) promoters of genes encoding lipoxygenases (e.g., Peng et al., J. Biol. Chem. 269: 3755-3761, 1994);

b) promoters of genes encoding peroxidases (e.g., Chittoor et al., Mol. Plant-Microbe Interactions 10: 861-871, 1997);

c) promoters of genes encoding hydroxymethylglutaryl-CoA reductase (e.g., Nelson et al., Plant Mol. Biol. 25: 401-412, 1994);

d) promoters of genes encoding phenylalanine ammonia lyase (e.g., Lamb et al., Abstract of the general meeting of the International Program on Rice Biotechnology, Malacca, Malaysia, Sept. 15-19, 1997);

e) promoters of genes encoding glutathione-S-transferase;

f) promoters from pollen-specific genes, such as corn Zmg13;

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g) promoters from genes encoding chitinases (e.g., Zhu & Lamb, Mol. Gen. Genet. 226: 289-296, 1991);

h) promoters from plant viral genes, either contained on a bacterial plasmid or on a plant viral vector (e.g., as described by Hammond-Kosack et al., Mol. Plant-Microbe Interactions 8: 181-185,1994);

i) promoters from genes involved in the plant respiratory burst (e.g., Groom et al., Plant J. 10(3): 515-522, 1996); and

j) promoters from plant anthocyanin pathway genes (e.g., Reddy, pp 341-352 in Rice Genetics III, *supra*; Reddy et al., Abstract of the general meeting of the International Program on Rice Biotechnology, Malacca, Malaysia, Sept. 15-19, 1997).

The coding region is also operably linked to an appropriate 3' regulatory sequence. In a preferred embodiment, the nopaline synthetase polyadenylation region is used. Other useful 3' regulatory regions include, but are not limited to the octopine polyadenylation region.

Using an *Agrobacterium* binary vector system for transformation, the selected coding region, under control of appropriate regulatory elements, is linked to a nuclear drug resistance marker, such as kanamycin resistance. Other useful selectable marker systems include, but are not limited to: other genes that confer antibiotic or herbicide resistances (e.g., resistance to hygromycin or bialaphos) or herbicide resistance (e.g., resistance to sulfonylurea, phosphinothricin, or glyphosate).

Plants are transformed and thereafter screened for one or more properties, including the presence of piNOS protein, piNOS-encoding mRNA, NO synthesizing activity, or enhanced resistance to plant pathogens or other disease-causing agents. It should be recognized that the amount of expression, as well as the tissue-specific

pattern of expression of the transgenes in transformed plants can vary depending on the position of their insertion into the nuclear genome. Such positional effects are well known in the art. For this reason, several nuclear transformants should be regenerated and tested for expression of the transgene.

As mentioned above, NO is a key component in the signal transduction pathway(s) leading to activation of certain defense responses in plants after pathogen attack (see Fig. 20 for a schematic depiction of central role postulated for NO in plant disease defense responses). The identification of piNOS in accordance with the present invention enables the identification of compounds that, through modulation of piNOS production or activity in plant cells, may contribute to the enhancement of disease defense responses in plants. Accordingly, the present invention features methods to identify such modulators of piNOS activity or production.

A typical method for identifying agents that modulate piNOS enzymatic activity is performed by combining a pre-determined amount of the piNOS (which may be in solution or affixed to a solid support) with a substrate acted upon by the piNOS to produce a product. Preferably, the substrate or the product, or both, are directly or indirectly detectable, the piNOS and its substrate are combined in the presence or absence of a test compound suspected of modulating the piNOS activity. Conversion of the substrate to the product in the presence and absence of the test compound is measured, with a change in rate or total amount of conversion of the substrate to the product in the presence of the test compound being indicative that the test compound modulates piNOS enzymatic activity. Such assays are well known to those of skill in the art, and are now possible due to the discovery of piNOS in accordance with the present invention. Moreover, the method is adaptable for

identifying agents that modulate piNOS enzymatic activity in host cells. In this embodiment, a piNOS-encoding gene is introduced into the host cells and the assay is performed by treating a sample of cells with a test compound and thereafter comparing piNOS enzymatic activity in treated and untreated cells.

Another assay in accordance with the present invention enables identification of agents that modulate expression of genes encoding piNOS in host cells. In this type of assay, a piNOS-encoding gene, comprising a coding sequence and expression regulatory sequences, is introduced into host cells. A sample of the host cells is treated with a test compound suspected of modulating genes encoding piNOS, and piNOS-encoding gene expression in the sample of host cells treated with the test compound is compared with gene expression in an equivalent sample of host cells not treated with the test compound. PiNOS-encoding gene expression may be measured in a variety of ways known to those of skill in the art, including determining the amount of (1) piNOS-encoding mRNA, (2) piNOS protein, and/or (3) piNOS enzymatic activity in the cells. Alternatively, the piNOS coding sequence can be replaced by a heterologous coding sequence encoding a detectable gene product, and the piNOS-encoding gene expression can be measured by measuring the detectable gene product.

The present invention also features methods to enhance resistance of a plant to plant pathogens or other disease causing agents, comprising increasing an amount or activity of a piNOS enzyme in the plant. In one embodiment, this may be accomplished by treating the plant with an up-regulator of piNOS enzymatic activity identified by one or more of the methods described above.

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Other embodiments utilize genetic engineering techniques. The potential of recombinant genetic engineering methods to enhance disease resistance in agronomically important plants has received considerable attention in recent years. Protocols are currently available for the stable introduction of genes into plants (as described in detail above), as well as for augmentation of gene expression. The present invention provides nucleic acid molecules that, upon stable introduction into a recipient plant, can enhance the plant's ability to resist pathogen attack. For instance, in one embodiment of the invention, a piNOS-encoding gene under control of its own expression-controlling sequences is used to transform plants for the purpose of increasing production of piNOS in the plants. Alternatively, a piNOS coding region is operably linked to heterologous expression controlling regions, such as constitutive or inducible promoters. In one embodiment, the promoter is either generally pathogen inducible (i.e. inducible upon challenge by a broad range of pathogens) or wound inducible. The chimeric gene is then used to transform the plant of interest. Upon wounding or challenge with a plant pathogen, the resulting transgenic plants would be induced to produce piNOS, thereby triggering a disease defense response. In any of the foregoing embodiments, the piNOS-encoding gene expression may be further enhanced by treating the plant with an up-regulator of piNOS-encoding gene expression or an activator of the enzymatic activity of piNOS identified by one or more of the screening assay methods described above.

For certain purposes, e.g., to further elucidate signal transduction pathways leading to the disease defense response in plants, it may be desirable to inhibit production or activity of piNOS, or to otherwise interfere with piNOS-mediated signal transduction. The present invention provides methods to accomplish these objectives.

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For instance, expression of piNOS-encoding genes or enzymatic activity of piNOS may be down-regulated by treating a plant with one or more of the modulating compounds identified by the assays described above.

Alternatively, piNOS loss-of-function (null) mutant plants may be created or selected from populations of plant mutants currently available. It will also be appreciated by those of skill in the art that mutant plant populations may also be screened for mutants that over-express *piNOS*, utilizing one or more of the methods described herein. Mutant populations can be made by chemical mutagenesis, radiation mutagenesis, and transposon or T-DNA insertions. The methods to make mutant populations are well known in the art.

The nucleic acids of the invention can be used to identify *piNOS* mutants in various plant species. In species such as maize or *Arabidopsis*, where transposon insertion lines are available, oligonucleotide primers can be designed to screen lines for insertions in the *piNOS* gene. Plants with transposon or T-DNA insertions in the *piNOS* gene are likely to have lost the function of the gene product. Through breeding, a plant line may then be developed that is homozygous for the non-functional copy of the *piNOS* gene. The PCR primers for this purpose are designed so that a portion of the coding sequence the *piNOS* gene are specifically amplified using the sequence of the *piNOS* gene from the species to be probed (see Baumann et al., 1998, Theor. Appl. Genet. 97: 729-734).

A plant also may be engineered to display a phenotype similar to that seen in null mutants created by mutagenic techniques. A transgenic null mutant can be created by a expressing a mutant form of the piNOS protein to create a "dominant negative effect." While not limiting the invention to any one mechanism, this mutant

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piNOS protein will compete with wild-type piNOS protein for interacting proteins or form an inactive dimer (or other multimer) with the wild-type piNOS protein in a transgenic plant. By over-producing the mutant form of the protein, the signaling pathway used by the wild-type piNOS protein or the activity of the dimeric piNOS can be effectively blocked. Examples of this type of "dominant negative" effect are well known for both insect and vertebrate systems (Radke et al, 1997, Genetics 145: 163-171; Kolch et al., 1991, Nature 349: 426-428). In a preferred embodiment, the mutant protein is produced by mutating the coding sequence corresponding to several residues in the amino-terminal region of piNOS.

Another kind of transgenic null mutant can be created by inhibiting the translation of the piNOS-encoding mRNA by "post-transcriptional gene silencing." The piNOS-encoding gene from the species targeted for down-regulation, or a fragment thereof, may be utilized to control the production of the encoded protein. Full-length antisense molecules can be used for this purpose. Alternatively, antisense oligonucleotides targeted to specific regions of the piNOS-encoded RNA that are critical for translation may be utilized. The use of antisense molecules to decrease expression levels of a pre-determined gene is known in the art. Antisense molecules may be provided *in situ* by transforming plant cells with a DNA construct which, upon transcription, produces the antisense RNA sequences. Such constructs can be designed to produce full-length or partial antisense sequences. This gene silencing effect can be enhanced by transgenically over-producing both sense and antisense RNA of the gene coding sequence so that a high amount of dsRNA is produced (for example see Waterhouse et al., 1998, PNAS 95: 13959-13964). In this regard, dsRNA containing sequences that correspond to part or all of at least one intron have

been found particularly effective. In one embodiment, part or all of the piNOS coding sequence antisense strand is expressed by a transgene. In another embodiment, hybridizing sense and antisense strands of part or all of the piNOS coding sequence are transgenically expressed.

Yet another type of synthetic null mutant can also be created by the technique of "co-suppression." Plant cells are transformed with a copy of the endogenous gene targeted for repression. In many cases, this results in the complete repression of the native gene as well as the transgene. In one embodiment, a piNOS-encoding gene from the plant species of interest is isolated and used to transform cells of that same species.

Mutant or transgenic plants produced by any of the foregoing methods are also featured in accordance with the present invention. Preferably, the plants are fertile, thereby being useful for breeding purposes. Thus, mutant or plants that exhibit one or more of the aforementioned desirable phenotypes can be used for plant breeding, or directly in agricultural or horticultural applications. They will also be of utility as research tools for the further elucidation of signal transduction pathways in plant disease defense responses. Plants containing one transgene or a specified mutation may also be crossed with plants containing a complementary transgene or genotype in order to produce plants with enhanced or combined phenotypes.

The following examples are provided to illustrate, not to limit, the invention.

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EXAMPLE 1

Purification of Nitric Oxide Synthase (NOS) Activity from Tobacco

This example sets forth detailed methods for purifying a NOS-like enzyme from tobacco, and results of the purification scheme.

Infection of tobacco with TMV: Tobacco plants *(Nicotiana tabacum* cv Xanthi nc (NN) and Xanthi (nn) were grown at 22°C in growth chambers programmed on a 14-hr light, 10-hr dark cycle. Four to six week old plants were used for experimentation. Xanthi nc (NN) were mechanically infected with TMV (1 μg/ml in 10 mM Hepes buffer [pH. 7.0] in the presence of carborundum) or buffer only while Xanthi (nn) was infected with TMV. Plants were shifted to 32°C 40 hr before infection. Defense responses were activated by shifting plants from 32°C to 22°C, 48 hr after infection. Samples were taken at different times and frozen in liquid nitrogen. Proteins were extracted and NOS activity was measured using a oxy-hemoglobin assay (see details below) as shown in Fig. 1A. The NOS induction was also monitored in plants grown and infected at 22°C. In this case the experiment was performed by using detached leaves. The secondary and tertiary detached leaves were mechanically infected with TMV (1 μg/ml) in 10 mM Hepes buffer (pH 7.0) in the presence of carborundum and floated on buffer soaked papers (Whatman 3MM) in plastic trays. The trays were covered with plastic wrap and kept at 22°C in a growth chamber programmed on a 14-hr light, 10-hr dark cycle. Samples were taken at different times and frozen in liquid nitrogen. Proteins were extracted and NOS activity was measured as shown in Fig. 1B. For purification of protein TMV infection was done by using whole plant, rather than infecting the detached leaves.

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Infection of tobacco with *Pseudomonas syringe pv. maculicola*: Leaves of four week old plants were infected at 22°C with *Pseudomonas* syringe pv. maculicola by infiltration of resuspended bacteria stock (OD_{600} ~ 0.2, i.e., 10^8 cells/ml) (leaves were not detached). Bacteria were resuspended shortly before infection as follows. *P. syringe pv. maculicola* were maintained on an agar plate containing King's medium (20 ml glycerol, 40 g peptone, 10 ml 10% K_2HPO_4, 10 ml 10% $MgSO_4$ in a volume of 1 liter and 50 μg/ml rifampicin). A 3 ml overnight culture of *P. syringe* was prepared in King's medium at 28°C with vigorous aeration and 1 ml was used to inoculate 50 ml of fresh medium. The culture was grown at 28°C with vigorous aeration to OD_{600} of ~ 0.2 (usually ~ 3 hr). Cells were harvested by centrifugation using table top swing out bucket centrifuge at 4500 rpm, 4°C for 5 minutes. Cells were washed by resuspension in 25 ml of 10 mM $MgCl_2$ and repelleting before resuspension in 10 mM $MgCl_2$ (cold) to a final OD_{600} of 0.2 and use immediately for infection. Infiltration of 10 mM $MgCl_2$ served as the mock control. NOS activity was measured at various in times, (details below) as shown in Fig. 2.

Purification of NOS: Tobacco leaves were infected with TMV at 22°C and infected plants were kept at 22°C for another 20-22 hr. The leaves were harvested and washed with distilled water, frozen in liquid nitrogen, and stored at –80°C. The purification protocol was started with at least 8-10 kg of tissue. All the glassware used in the process was pre-cooled to 4°C and all the subsequent steps were carried out at 4°C. The frozen leaves were ground with liquid nitrogen into a fine powder using mortar and pestle and proteins were extracted using an extraction buffer (20 mM Hepes, 2 mM EDTA, 5 mM EGTA, 5 mM DTT or 5 mM mercaptoethanol, 2 mM aprotonin, 4 μM FAD, 2 mM phenylmethanesulfonyl fluoride (PMSF), 1%

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polyvinypolypyrrolidone (PVPP) and 10% glycerol). The extract was centrifuged at 12,000 rpm in a GSA (Sorvall) rotor for 60 minutes at 4°C. The rotor and centrifuge bottles were pre-cooled at 4°C. The supernatant was collected and used for further purification. NOS activity and protein concentration were measured at each step.

G-25 Sephadex Chromatography: Sephadex G-25 beads were hydrated in H_2O (1 gm in 4-6 ml) for minimum 10-12 hr. Once the beads were fully swollen, they were washed thoroughly in H_2O and then equilibrated with equilibration buffer (10 mM Hepes, 2 mM EDTA, 5 mM EGTA, 5 mM DTT or 5 mM mercaptoethanol, 2 mM aprotonin, 4 μM FAD, 2 mM PMSF and 10% glycerol). The equilibrated beads were packed (250 ml) into a chromatography column (Pharmacia, 70 cm x 4.5 cm). The supernatant from the step above was loaded on to this Sephadex G-25 column and proteins were eluted using the equilibration buffer. NOS activity appearing in the void volume was measured and proteins were processed for the next step of purification, differential ammonium sulfate precipitation. Four columns were run simultaneously.

Ammonium Sulfate Precipitation: Powdered ammonium sulfate was slowly added to a final concentration of 30-35% saturation to a continuously stirring of G-25 Sephadex void volume eluted NOS activity. The slurry was stirred for an addition 30 minutes after the final addition of ammonium sulfate and was followed by centrifugation at 10,000 rpm in a GSA rotor at 4°C. The pellet was resuspended in 80 ml of equilibration buffer and dialyzed against the equilibration buffer with 4-5 changes (total buffer of 10-12 liters) over 4-5 hr. The dialyzed sample was subjected to DEAE-Sepharose chromatography.

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DEAE-Sepharose Chromatography: DEAE-Sepharose suspension was mixed and washed thoroughly with distilled water (minimum of 10-12 times of the volume used). The washed beads were equilibrated with equilibration buffer. The equilibrated beads (200 ml) were mixed with proteins obtained after ammonium sulfate fractionation and were rotated overnight on a table top shaker (15-20 rpm) at 4°C for batch absorption. The matrix was packed into a chromatography column (Pharmacia, 90 cm x 4 cm) at a flow rate of 80 ml/hr. Protein eluate (flow through) was collected and recirculated 2-3 times at a flow rate of 20 ml/hr. The column was washed with washing buffer (10 mM Hepes, 2 mM EDTA, 5 mM EGTA, 5 mM DTT or mercaptoethanol, 2 mM aprotonin, 4 μM FAD, 2 mM PMSF, 10% glycerol and 2 μM (6R)- tetrahydro-L-biopterin (H_4B)), using a minimum of 5 column volumes. Proteins were eluted with elution buffer (10 mM Hepes, 2 mM EDTA, 5 mM EGTA, 5 mM DTT or mercaptoethanol, 2 mM aprotonin, 4 μM FAD, 2 mM PMSF, 10% glycerol , 2 μM H_4B, 100 μM arginine) with a linear NaCl gradient from 0-0.5 M in a total volume of 300 ml at a flow rate of 30 ml/hr. Five ml fractions were collected. NOS activity and the total protein profiles are shown in Fig. 3. Fractions containing peak NOS activity (#3-15) were pooled and reverse dialyzed on sucrose bed at 4°C to reduce the volume to 1/3 and was dialysed against equilibration buffer for 3-4 hr total with 3-4 changes. The dialyzed sample was subjected to next step of purification, ARG-Sepharose.

ARG-Sepharose Chromatography: 25 ml of arginine-Sepharose beads were washed with 1 liter of distilled water and equilibrated with equilibration buffer. The equilibrated beads were packed in a chromatography column (BioRad 32 cm x 2 cm)

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at a flow rate of 50 ml/hr. Pooled fractions containing peak NOS activity from DEAE-Sepharose chromatography were loaded on to the ARG-Sepharose column with a flow rate of 3 ml/hr and recirculated 3-4 times (0.1 ml/min). The column was washed with washing buffer till absorbance at 280 reached zero and proteins were eluted with washing buffer containing100 μM L-arginine and a linear gradient of NaCl (0-0.5 M) in total volume of 100 ml. Four-ml fractions were collected. Fig. 4 shows the NOS activity and the total protein profiles and Fig. 5 shows the level of NOS activity and SDS-PAGE of the proteins from fractions containing the majority of the NOS activity. Fractions with peak NOS activity (#9-15) were pooled and subjected to ADP-Sepharose chromatography.

ADP-Sepharose Chromatography: ADP-Sepharose beads were hydrated in distilled water (1 g in 4 ml) for 10-12 hr and were washed several times with distilled water. The washed beads were equilibrated with equilibration buffer and were packed (16 ml) in a Biorad column (32 cm x 2 cm) at a flow rate of 50 ml/hr. Pooled fractions containing peak NOS activity from ARG-Sepharose chromatography were diaylsed against equilbration buffer 1.5 liter (3-4 changes) loaded with a flow rate of 5 ml/hr and recirculated through the column at least 3-4 times (0.1 ml/min). The column was washed with washing buffer (10 mM Hepes, 2 mM EDTA, 5 mM EGTA, 5 mM DTT or 5 mM mercaptoethanol, 2 mM aprotonin, 4 μM FAD, 2 mM PMSF, 10% glycerol and 2 μM H_4B) with a flow rate of 30 ml/hr until absorbance at 280 reached zero. Protein was eluted with washing buffer containing 100 μM arginine and a linear gradient of NaCl (0-1.0 M) in a total volume of 200 ml. Five ml fractions were collected. Fig. 6 shows the resulting NOS activity and total protein

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profiles and Fig. 7 shows the level of NOS activity and SDS-PAGE of the protein from fractions containing the majority of the NOS activity. Proteins on the SDS-PAGE were visualized by silver staining. Fractions with peak NOS activity (#3-9) were loaded to a CaM-Sepharose column.

CaM-Sepharose Chromatography: Pooled fractions containing peak NOS activity from the ADP-Sepharose chromatography step were reverse dialyzed on a sucrose bed at 4°C to reduce volume to half, followed by dialysis against 1 liter of equilibration buffer (minus EGTA and EDTA) for 2-3 hr with three changes. Calcium chloride was added to the dialyzed sample to 0.1 mM and the sample was then rotated on a table top shaker (15-20 rpm) at 4°C for 1-2 hr. CaM-Sepharose beads (15 ml) were washed thoroughly with distilled water and equilibrated with CaM-Sepharose column buffer (20 mM Tris [pH, 7.0], 1 mM imidazole, 1 mM magnesium acetate, 0.01 mM $CaCl_2$, 10 mM mercaptoethanol, 4 μM FAD, 5 mM PMSF and 10% glycerol) followed by packing of the beads into a chromatography column (BioRad 32 cm x 1.2 cm) with a flow rate of 40 ml/hr. The dialyzed sample, containing 0.1 mM $CaCl_2$ was loaded on to the CaM-Sepharose column with a flow rate of 12 ml/hr and was recirculated 2-3 times (0.1ml/min). After loading, the column was washed with CaM-Sepharose column buffer, followed by a wash with CaM-Sepharose column buffer (100 ml) supplement with 0.05 M NaCl. Proteins were eluted with CaM-Sepharose elution buffer (20 mM Tris [pH, 7.0], 1 mM magnesium acetate,10 mM mercaptoethanol, 4 μM FAD, 5 mM PMSF, 2 μM H_4B , 100 μM arginine, 200 mM NaCl, 10% glycerol and a linear gradient of EGTA (0-5.0 mM) at a flow rate of 20 ml/hr in a total volume of 50 ml. Two ml fractions were collected. Fig. 8 shows the NOS activity and total protein profiles of fractions from

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CaM-Sepharose chromatography. Fig. 9 shows the level of NOS activity and SDS-PAGE of the proteins from CaM-Sepharose eluted fractions.

Results: A comparison of NOS activity in crude extract and pooled peak NOS activity fractions from each step of the purification using two different methods of measurements, oxy-hemoglobin assay and citrulline assay (details in Example 2), is shown in Fig. 10. Fig. 11 shows the analysis of these fractions by SDS-PAGE. Table 1 below summarizes the results of the NOS purification scheme, resulting in a ~33,000-fold purification of the NOS activity from tobacco.

Fraction	Protein (mg)	Total Activity (pmol/min)	Specific Activity (pmol/min/mg)	Fold	Recovery (%)
Crude	16,000	480,000	30	1	100
$(NH_4)_2SO_4$	842	320,000	380	13	66.6
DEAE-Sepharose	49	291,520	5,954	198	60.7
ARG-Sepharose	1.9	89,120	46,905	1,564	18.6
ADP-Sepharose	0.8	63,510	79,388	2,646	13.2
CaM-Sepharose	0.02	19,674	983,700	32,790	4.1

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EXAMPLE 2

Characterization of Tobacco Nitric Oxide Synthase

This example describes the characterization of the polypeptide(s) exhibiting NOS activity, purified from tobacco as described in Example 1.

Fluorescence and inhibitor studies: Tryptophan (Fig. 12) and flavin fluorescence (Fig. 13) changes of the tobacco NOS were recorded by using a SLM 8000 spectrofluorometer. The purified NOS from the CaM-Sepharose column was diluted to 2 μg/ml in 10 mM Hepes (pH 7.0) in 0.5 ml quartz cuvette. Measurements (at 25°C) were initiated by irradiating the protein at 288-300 nM for tryptophan fluorescence and 450-460 nM for flavin fluorescence. The emission spectra were recorded at 300-390 nM for tryptophan and 500-600 nM for flavin, before and after the addition of $CaCl_2$ (100 μM) in presence of different concentrations of CaM (0.1-2.0 μM) to induce the formation of calmodulin-NOS complex and addition of EDTA (200 and 800 μM) to induce dissociation of the complex. The mixture was stirred in the cuvette for 5 minutes after each addition.

The sensitivity of the highly purified tobacco NOS to different concentrations (0-50 μM) of two inhibitors of animal NOS, N(G)-monomethyl-L-arginine (L-NMMA) and aminoguanidine, were tested (Fig. 14). NOS activity was monitored by oxy-hemoglobin assay. The inhibitors were added before incubation of the reaction mixture at 37°C.

NOS activity assays: The oxy-hemoglobin assay is based on a direct reaction between NO and the oxygenated, ferrous form of hemoglobin (HbO_2) which yields

the ferric form, methemoglobin (metHb) and nitrate. The rate of NO production arising from NOS activity is quantified by measuring the conversion of HbO_2 to metHb. To ensure that metHb production accurately measured NO synthesis in our assay, metHb production was also monitored in the presence of nitro-L-arginine. This arginine analog inhibits NO synthesis; therefore any metHb production in its presence, due to non-specific HbO_2 oxidation, was subtracted from the level of metHB produced in the absence of this inhibitor. A reaction mixture (total volume of 1 ml) in 10 mM Hepes (pH, 7.0) contained enzyme, (200 µl from crude or G-25 Sephadex or 100 µl from rest of the steps of purification), 1 mM arginine or 100 µM N-hydroxy-L-arginine (NOHA) or 1 mM nitro-L-arginine, 1 mM magnesium diacetate, 1 mM $CaCl_2$, 1 µM CaM, 4 µM FAD and was incubated at 37°C for 30 minutes. The reaction was started by addition of NADPH to 100 µM and H_4B to 10 µM. Change in absorbance was recorded at 401 nM. Direct contact between HbO_2 and H_4B should be avoided and HbO_2 should be in the ferrous form.

Citrulline Assay: The citrulline assay was done according to the procedure of Bredt and Synder (Proc. Natl. Acad. Sci. USA 86: 9030-9033, 1989).

Protein Estimation and SDS-PAGE Analysis: Protein profile of fractions was measured by recording absorbance at 280 nm while for specific activity determination, protein was quantified by using Bradford's procedure. SDS-PAGE analyses of proteins at different steps of purification was done by electrophoresis on a 7.5 – 10 % polyacrylamide gel by using Laemmli's protocol followed by silver staining to visualize the proteins. Table 2 presents a summary of various biochemical features of tobacco NOS, as compared with animal NOSs.

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Table 2: Comparison of animal NOSs with those of the tobacco NOS

Properties	Animal NOSs	Tobacco NOS
Size	130-160 KDa	120 Kda
Dependency on H_4B, FAD, FMN, NADPH	+	+
Ca^{2+} requirement	+	+
CaM requirement	+	+
Inhibition by L-NMMA	+	+
Inhibition by aminoguanidine	+	+
Change in flavin fluorescence with CaM	+	+
Change in tryptophan fluorescence with CaM	+	+
NO synthesis detected by citrulline formation assay	+	+
NO synthesis detected by oxy-hemoglobin assay with either Arg or NOHA as substrate	+	+

EXAMPLE 3

Characterization of *Arabidopsis* Gene Encoding an Enzyme Having NOS Activity

Four different peptide sequences were obtained by MS-Q(TOF)/MS-MS analysis of polypeptide co-purifying with NOS-like activity. The sequence analysis of these four peptides using *Arabidopsis* database corresponded to a putative P subunit of glycine decarboxylase. Fig. 15A shows the deduced amino acid sequence of the *Arabidopsis* candidate P subunit (*CanP*) of glycine decarboxylase (SEQ ID NO:1). The comparison of *CanP* with animal NOSs was done and Fig. 15B shows

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the critical motifs of the *Arabidopsis* variant P subunit, including those shared with mammalian NOSs.

Sequence analysis indicated the presence of NADPH binding-Rossmann fold domain in *CanP* variant; NADPH binding Rossmann fold domain has been shown to bind tetrahydrofolate (THF). Tetrahydrofolate is also a biopterin and can function similarly to tetrahydrobiopterin (H_4B) with some NOSs. Therefore, the NOS-like enzyme activity was compared with THF and H_4B using CaM-sepharose purified fraction in presence of L-arginine and N-hydroxy-L-arginine (NOHA) as substrates with the oxy-hemoglobin assay as described before. The reaction mixture contained 25 µl of enzyme, $CaCl_2$ (1 mM), CaM (1 µM), H_4B/THF (10 µM), NADPH (100 µM), FAD (4 µM), magnesium diacetate (1 mM) and hemoglobin in 20 mM Hepes (pH 7.0). Fig. 16 shows that H_4B can be replaced by THF.

Further confirmation that the variant P subunit has NOS-like activity was obtained by demonstrating the inhibition of NOS-like activity carboxymethoxylamine (CM), an inhibitor of P subunit of glycine decarboxylase. The reaction mixture contained 25 µl of CaM-sepharose purified enzyme, $CaCl_2$ (1 mM), CaM (1 µM), H_4B (10 µM), (+/-) NADPH (100 µM), (+/-) pyridoxal phosphate (PP) (20 µM), FAD (4 µM), magnesium diacetate (1 mM), (+/-) CM (200 µM) and hemoglobin in 20 mM Hepes (pH 7.0). The NOS-like activity was monitored by using arginine and NOHA as substrates with the oxy-hemoglobin assay as described before. Fig. 17 shows that NOS-like activity was inhibited by CM, and that PP can partially substitute for NADPH or a cofactor for NOS-like activity.

Changes in NOS-like activity in *Arabidopsis* ecotypes after TCV infection:

The *Arabidopsis* ecotypes, susceptible (Col-0) and resistant (Di-17) were grown in

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chambers at 5000 LUX with a 16 hr photoperiod at about 50% humidity both before and after inoculation. Three weeks old plants containing at least four fully expanded leaves were used for inoculation. Turnip crinkle virus (TCV) is inoculated as a positive strand RNA produced by *in vitro* transcription (Dempsey et al., Phytopathology 83: 1021-1029). The inoculation medium contains 100 µl diethyl-pyrocarbonate (DEPC) treated water, 100 µl 2X autoclaved inoculation buffer (50 mM glycine, 30 mM K2HPO4, 0.02% Bentonite, 1% celite, pH 9.2) and 2.5 µg RNA. Inoculation was done using a glass rod and spatula (glass rod and spatula were treated with 10% bleach for 15 min and washed with DEPC treated water before use). The spatula is used as support underneath the leaf and the glass rod containing inoculum is rubbed gently on the upper surface. The mock treatment was done by rubbing inoculation medium minus RNA. After infection, the tissues was frozen in liquid nitrogen at different times and proteins were extracted using the NOS extraction buffer 1:2 vol (as described before). The extract was centrifuged at 13,000 rpm, SS34 (Sorvall) rotor for 45 minutes at 4°C. The rotor and centrifuge tubes were pre-cooled at 4°C. The supernatant was collected and passed to G-25 Sephadex column (2 ml), pre-equlibrated with equilibration buffer. Protein and NOS activity was monitored in void volume fraction as described earlier. Fig. 18 shows the changes in NOS activity in susceptible (Col-0) and resistant (Di-17) *Arabidopsis* ecotypes. NOS activity was induced in resistant (Di-17) ecotype after 24 hrs of infection with TCV. In contrast, this activity was not induced in a TCV-infected susceptible ecotype (Col-0).

Cloning variant P (*CanP*) in pET-28a and pFastBac plasmids: A full length cDNA clone (SEQ ID NO:6) was obtained from *Arabidopsis* Biological Resource Center (ABRC). The complete fragment was amplified by using

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oligonucleotides GGATCCATGGAGCGCGCAAGGAGACTTGC (SEQ ID NO:7) and GTCGACTCAAGCAGACACTGCAGCTGCGAC (SEQ ID NO:8) which have BamH I and Sal I sites, respectively, using Herculase hotstart DNA polymerase (Stratagene). The PCR was done using 2 min 92°C, 10 sec 92°C, 30 sec 58°C, 3.5 min 72°C, 1 min 72°C for 30 cycles. The amplified fragment was run on 0.8% agarose in Tris-acetate-EDTA buffer (1x TAE) gel containing ethidium bromide and was excised and purified using a gel extraction kit from Qiagene.

Vector, pET-28a (100 ng) was used for expressing *CanP* in *E. Coli* and vector pFastBac (100 ng) was used for expressing *CanP* in baculovirus-infected insect cells. Vectors and purified fragment (1 μg) were digested with BamH I and Sal I at 37°C for 2-3 hrs. Digestion was checked on 0.8% agarose gel (1x TAE) and digested products were purified using Qiagene kit. 30 ng of pET-28a and pFastBac vectors were ligated with 300 ng of digested fragment at 16°C using T4 DNA ligase overnight. The fragment ligated with pET-28a vector was transformed into DH5x competent cells using electroporation. For electroporation transformation protocol, 10 μl of ligated mixture was mixed with 20 μl of ethanol and 6 μl of 3 M sodium acetate (pH 5.2) and was incubated at –70°C for 1 hr followed by centrifugation at 4°C for 15 min. The pellet was washed with 70% ethanol, air dried for 5 min and was mixed with 10 μl of commercial competent cells. The mixture was incubated in electroportaion chamber on ice in electroporation cuvette and electroporation was done by setting the position at 410 volt DC. Transformed cells were mixed with 1 ml of TB medium and were incubated at 37°C for 30 min. The mixture was spread on kanamycin containing (50 μg/ml) plates. The plates were incubated at 37°C for 12-16 hr. Colonies were screened for positives by using PCR reaction as described above and by isolating

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plasmid using Qiagene kit, followed by digestion with BamH I and Sal I. Digested product was checked on 0.8% agarose (1X TAE). Colonies containing an insert were used for production of protein expression in *E. Coli.*

The *CanP* fragment ligated to pFASTBac was transformed into DH10BacTMcells. 100 µl of competent cells were thawed on ice and were mixed gently with 10 µl of ligated mixture. The mixture was incubated on ice for 30 min, heat shocked at 42°C for 45 sec and was chilled on ice for 2 min. 800 µl of LB medium was added to the mixture and incubation was done at 37°C with agitation (225 rpm) for 4 hrs. Serial dilutions of the mixture (10^{-1} and 10^{-2}) were spread on plates containing kanamycin (50 µg/ml), gentamycin (7 µg/ml), tetracycline (10 µg/ml), X-gal (100 µg/ml) and isoporopylthio-ß-D-galatoside (IPTG) (40 µg/ml). Plates were incubated at 37°C for 24-48 hrs. White colonies contained the recombinant bacmid and therefore were selected for isolation of recombinant bacmid DNA. Before isolating DNA, candidate colonies were streaked again on plates containing kanamycin (50 µg/ml), gentamycin (7 µg/ml), tetracycline (10 µg/ml), X-gal (100 µg/ml) and IPTG (40 µg/ml) to ensure they were truly white. A few white colonies were picked and from a single white colony, a liquid culture (2 ml) containing kanamycin (50 µg/ml), gentamycin (7 µg/ml), tetracycline (10 µg/ml) antibiotics was set up for isolation of bacmid DNA. Cells were grown at 37°C to stationary phase (up to 24 hr) with shaking at 250-300 rpm. 1.5 ml of culture was transferred into microcentrifuge tube and was centrifuged at 14,000 x g for 1 min. Supernantant was removed and the pellet was suspended by gentle pipetting in 0.3 ml of solution I (15 mM Tris-HCl {pH 8.0}, 10 mM EDTA, 100 µg/ml RNase A). 0.3 ml of solution II (0.2 N NaOH, 1% SDS) was added and was mixed gently. The

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mixture was incubated at room temperature for 5 min. 0.3 ml of 3 M potassium acetate was added to the tube and was mixed gently again and was incubated on ice for 5 min. The mixture was centrifuged at 14,000 x g for 10 min at room temperature and supernatant was transferred to a new tube, followed by addition of 0.8 ml of isopropanol and overnight incubation at –20°C. The mixture was centrifuged at 14,000 x g at room temperature for 15 min and the pellet was washed with 70% ethanol and finally air dry for 10-15 min at room temperature. DNA was dissolved in 40 µl of TE and was checked on agarose gel 0.8% (1X TAE), then used for transfection of Sf9 insect cells.

***CanP* expression in *E. coli*:** A single colony containing the *CanP* gene in pET-28a vector was inoculated in 1.5 ml of LB medium containing kanamycin (50 µg/ml) and was rotated on shaker 250-300 rpm at 37°C overnight. 100 µl of overnight grown cells were reinoculated in 10 ml of LB containing kanamycin (50 µg/ml) and were grown at 37°C overnight on shaker (250-300 rpm). Ten ml of overnight grown culture was transferred into 200 ml LB containing kanamycin (50 µg/ml) and grown at 37°C on ashaker (250-300 rpm) until the OD_{600} reached 0.4-0.6. IPTG (1 mM) was added and cells were transferred to 27°C for 6 hrs. No IPTG was added in the control. In order to obtain good quantities, 5 flasks of 200 ml induced culture were used for purification of protein.

Cells from induced and uninduced cultures (2ml) were pelleted by centrifugation at 4°C at 14,000 x g for 10 min, then suspended in 50 µl of 1X SDS sample buffer and incubated in boiling water for 5 min. The lysate was centrifuged and 5 µl of supernatant was run on a 10 % SDS-polyacrylamide gel for analysis of proteins in induced and uninduced cultures. The gel was stained with 0.05%

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Coomassie in 40% methanol and 10% acetic acid for 30 min at room temperature. The bands were visualized by destaining the gel in 40% methanol and 10% acetic acid. For measuring NOS activity in induced and uninduced cultures, the cells from 5 ml culture were lysed by sonication in native buffer (50 mM Na_2HPO_4,{pH 7.5}, 4 μM FAD, 5% glycerol, 5 mM ME ,100 μM aprotonin) on ice and the mixture was centrifuged at 4°C for 30 min at 14,000 x g. Supernatant was used for monitoring the NOS activity. Before measuring NOS activities, heme addition was done according to Hemmens et al. (1998) (Biochem. J., 332: 337-342). Sodium phosphate (50 mM), pH 7.5 was thoroughly degassed and D,L, dithiothreitol (DTT) was added to a final concentration of 60 mM. Crude induced and uninduced extracts (200 μl) were added to the above mixture along with 2 μl of 200 μM solution of heme. The solution was incubated at 20°C for 40 min. Then 2 μl of 10 mM (+/-) H_4B and 2 μl of 100 mM L-arginine or NOHA was added and NOS activities were monitored after 40 min of incubations in a reaction mixture containing 2 mM EDTA, 5 mM EGTA, 4 μM FAD, 1 mM magnesium diacetate, 1 mM $CaCl_2$, 1 μM CaM using oxy-hemoglobin assay, as described before. No heme was added to the control. The results are shown in Table 3.

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Table 3: NO synthesizing activity of the Arabidopsis *canP*-encoded protein in *E.coli* or baculovirus-infected Sf9 cells and its dependence on its cofactors H_4B and heme

	-H_4B		+H_4B		+H_4B+Heme	
E.coli Expt #1	ARG	NOHA	ARG	NOHA	ARG	NOHA
Uninduced	26[a]	44	44	44	44	44
Induced	158	255	746	965	1982	2720
E.coli Expt#2						
Uninduced	26	158	18	70	70	114
Induced[b]	158	307	509	1298	1184	2500
Ni column fractions[c]						
1	53	237	1132	2421	2579	4789
2	316	26	921	1711	2132	4000
3	79	737	737	1105	1553	2684
4	211	395	500	921	1395	2526
5	132	342	395	737	1526	2579
Sf9 cells[d]						
Mock	26	61	18	114	53	123
Empty Vector	0	70	0	9	0	26
canP	96	219	272	684	456	833

[a] NO synthesizing activity was determined with an oxy-hemoglobin assay and is expressed in pmol/mg/min.
[b] Expression of canP was induced with 1 mM IPTG and crude total extracts were prepared 6hr later.
[c] The His_6-tagged recombinant canP from the soluble fraction extracted from the IPTG-induced *E.coli* of Expt#2 was purified on a Ni^+ column.
[d] Sf9 insect cells were mock infected or infected either with recombinant baculovirus not carrying canP (empty vector) or carrying it (canP). Extracts were prepared at 72 hpi.

RG-Sepharose and ADP-Sepharose affinity columns. Cells were pelleted from one liter of culture by centrifugation in GSA rotor at 10,000 x g at 4°C for 30 min. The pellet was suspended in native buffer (50 mM Na_2HPO_4 [pH 7.5], 4 μM

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FAD, 5% glycerol, 5 mM ME ,100 μM aprotonin) and incubated at 30°C for 15 min with 50 μl of 10 mg/ml lysozyme. The suspension was sonicated on ice with 4x25 bursts and the lysate was centrifuged at 14,000 x g for 30 min in SS34 rotor. The lysate was used as starting material for purification using Ni-NTA His binding resin and ARG-Sepharose.

Ni-NTA His binding resin protocol: Resin (1.5 ml) was mixed with (50 mM Na_2HPO_4 [pH 7.5], 4 μM FAD, 5% glycerol, 5 mM ME ,100 μM aprotonin, 200 mM NaCl). The resin was allowed to settle and the supernatant was removed. This procedure was repeated two times. Lysate from the above step was mixed with NaCl (final concentration 200 mM) and was added to the resin and mixed gently by shaking (200 rpm on a rotatory shaker) at 4°C for 60 min. The mixture was loaded into a column and flow through was collected. The column was washed 2 x 6 ml with buffer (50 mM Na_2HPO_4,[pH 7.5], 4 μM FAD, 5% glycerol, 5 mM ME ,100 μM aprotonin, 1 M NaCl, 20 mM imidazole). Proteins were eluted with elution buffer (50 mM Na_2HPO_4 [pH 7.5], 4 μM FAD, 5% glycerol, 5 mM ME ,100 μM aprotonin, 500 mM NaCl and a gradient of EDTA (0-5 mM). NOS activities were monitored in eluted fraction after addition of heme, as described above.

ARG-Sepharose and ADP-Sepharose protocol: Lysate from induced culture (1 liter) was prepared as described above and was mixed with ARG-Sepharose beads (5 ml) equilibrated in equilibration buffer. The mixture was rotated on a shaker at 4°C for 4 hrs at 200 rpm and loaded into column with a flow rate of 3 ml/hr and recirculated 3-4 times (0.1 ml/min). The column was washed with washing buffer until absorbance at 280 nm reached zero, then proteins were eluted with washing buffer (2.5 ml) containing 100 μM L-arginine, 2 μM H_4B and 150 mM NaCl. NOS

activity was measured after addition of heme, as described above. The eluent was diaylsed against equilibration buffer (1 liter) for 1 hr at 4°C. The diaylsed sample was mixed with 2 ml of ADP-Sepharose beads pre-equlibrated with equilibration buffer in similar way as described above for NOS purification from tobacco extract. The column was washed with washing buffer until absorbance at 280 nm reached zero and proteins were eluted with washing buffer (1.5 ml) containing 100 μM arginine, 2 μM H_4B and 150 mM NaCl. NOS activity was measured after addition of heme, as described before. Fig. 19 shows the SDS-PAGE analysis of crude extracts from *CanP*-transformed *E. coli* or soluble fraction before or after purification by Ni+, ARG or ADP chromatography.

***CanP* Expression in Baculovirus-Transfected Target Cells.** Recombinant Bacmid containing *CanP* fragment was used for transfection of Sf9 cells. 9×10^5 Sf9 cells in 6 well plates were allowed to attach for at least 1 hr. The medium was removed from wells by tipping the plate slightly and pipeting carefully. Then 600 μl of the Grace's media and 10% fetal bovine serum (FBS) was added. Slowly 600 μl of transfection buffer (25 mM Hepes [pH 7.1], 140 mM NaCl, 125 mM $CaCl_2$) containing 5 μl of recombinant Bacmid DNA was added to the wells. Vector alone and Sf9 cells (mock) were kept as control. The plate was incubated at 27°C for 4 hrs and transfection solution was replaced with 1.5 ml TNMFH (Grace's medium [469.5 ml] containing fetal bovine serum heat inactivated [55 ml], lactalbumin [11 ml of 50x], yeastolate solution [11 ml of 50x, penicillin (50 units/ml)/streptomycin (50 μg/ml), 1.1 ml fungizone (final conc. 0.5 μg/ml)] in total of 550 ml) and allowed to incubate for 72 hrs at 27°C. Cells were checked under microscope after transfection and were scraped for harvesting the virus. The virus was harvested by centrifuging

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the mixture at 500 x g for 5 min at room temperature. Supernatant was stored as virus stock and cells were frozen for NOS activity analysis. 100 µl of viral stock was mixed with fresh 1.5 ml of TNMFH and was propagated for another 72 hr in infected cells, which were then harvested by centrifugation and lysed in native buffer (50 mM Na2HPO4 [pH 7.5], 4 µM FAD, 5% glycerol, 5 mM ME ,100 µM aprotonin) by sonication. Lysate was centrifuged at 4°C for 30 min at 14,000 x g. NOS activity was monitored in supernatant after addition of heme, as described before. Table 3 shows the comparison of NO synthesizing activity of the recombinant *Arabidopsis CanP* expressed in *E. Coli* or baculovirus-infected Sf9 cells.

While certain preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications may be made to the invention without departing from the scope and spirit thereof as set forth in the following claims.

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We claim:

1. An isolated plant enzyme having nitric oxide synthase (NOS) activity, wherein the NOS activity is inducible in a plant and comprises production of nitric oxide from arginine.

2. The enzyme of claim 1, wherein loss of the NOS activity of the enzyme in a plant results in altered resistance of the plant to plant pathogens or other disease-causing agents.

3. The enzyme of claim 1, wherein the NOS activity is characterized by one or more features selected from the group consisting of:

a) inducible by a plant pathogen;

b) having activity dependent on H_4B, FAD, FMN and NADPH;

c) requiring Ca^{2+} for activity;

d) requiring calmodulin for activity; and

e) synthesizing NO as determined by a citrulline formation assay or by an oxy-hemoglobin assay.

4. The enzyme of claim 3, wherein the NOS activity is further characterized by one or more features selected from the group consisting of:

a) activity inhibited by L-NMMA;

b) activity inhibited by aminoguanidine;

c) exhibiting calmodulin-mediated change in flavin fluorescence; and

d) exhibiting calmodulin-mediated change in tryptophan fluorescence.

5. The enzyme of claim 1, isolated from tobacco.

6. The enzyme of claim 5, having an apparent molecular mass of about 120 kDa as determined by SDS-PAGE.

7. The enzyme of claim 5, isolated from tobacco inoculated with TMV.

8. The enzyme of claim 7, isolated by a method comprising the steps of:

a) obtaining a crude extract of TMV-inoculated tobacco leaves;

b) subjecting the crude extract to G-25 Sephadex chromatography and collecting a void volume obtained therefrom;

c) subjecting the void volume to ammonium sulfate precipitation and collecting the 30-35% of saturation ammonium sulfate precipitate therefrom;

d) subjecting the dissolved 30-35% ammonium sulfate precipitate to DEAE-Sepharose chromatography and collecting NOS activity-containing eluate therefrom; and

e) subjecting the NOS activity-containing eluate from the DEAE-Sepharose chromatography to Arg-Sepharose chromatography and collecting NOS activity-containing eluate therefrom.

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9. The enzyme of claim 8, isolated by a method that comprises an additional step of:

f) subjecting the NOS activity-containing eluate from the Arg-Sepharose chromatography to ADP-Sepharose chromatography and collecting NOS activity-containing eluate therefrom.

10. The enzyme of claim 9, isolated by a method that comprises an additional step of:

g) subjecting the NOS activity-containing eluate from the ADP-Sepharose chromatography to CaM-Sepharose chromatography and collecting NOS activity-containing eluate therefrom.

11. Antibodies immunologically specific for part or all of the enzyme of claim 1.

12. The antibodies of claim 11, immunologically specific for an amino-terminal portion of the enzyme of claim 1.

13. The enzyme of claim 1, having an amino acid sequence comprising one or more fragments selected from the group consisting of

FGVPMGYGGPHAAFLATSQEYKR (SEQ ID NO:2);

GNINIEEVRK (SEQ ID NO:3);

IAILNANYMAK (SEQ ID NO:4); and

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GNADVQNNVL (SEQ ID NO:5).

14. The enzyme of claim 13, isolated from tobacco.

15. The enzyme of claim 13, having amino acid SEQ ID NO:1.

16. The enzyme of claim 15, produced by expression of an isolated nucleic acid molecule having at least 90% sequence identity with SEQ ID NO:6.

17. The enzyme of claim 15, produced by expression of an isolated nucleic acid molecule having at least 60% sequence identity with a portion of SEQ NO:6 encoding an amino-terminal portion of the enzyme of claim 1 comprising about 80 residues.

18. The enzyme of claim 15, produced by expression of an isolated nucleic acid molecule that hybridizes with the complement of SEQ ID NO:6 under hybridization conditions comprising:

hybridization at least six hours at 42°C in 5X SSC, 5X Denhardt's reagent, 1.0% SDS, 100 μg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide; and

washing at least once at room temperature in 2X SSC and 0.1 - 1% SDS with a final wash at 65°C in 0.1X SSC and 0.1% SDS for 10 minutes.

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19. The enzyme of claim 15, produced by expression of an isolated nucleic acid molecule that hybridizes with the complement of a portion of SEQ ID NO:6 encoding an amino-terminal portion of the enzyme of claim 1 comprising about 80 residues, under hybridization conditions comprising:

hybridization at least six hours at 42°C in 5X SSC, 5X Denhardt's reagent, 1.0% SDS, 100 μg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide; and

washing at least once at room temperature in 2X SSC and 0.1 - 1% SDS with a final wash at 45-55°C in 2X SSC and 0.1% SDS for10 minutes.

20. The enzyme of claim 15, produced by expression of an isolated nucleic acid molecule comprising SEQ ID NO:6.

21. An isolated nucleic acid molecule having a coding sequence that encodes a plant enzyme having nitric oxide synthase (NOS) activity, wherein the NOS activity is inducible in a plant and comprises production of nitric oxide from arginine.

22. The nucleic acid molecule of claim 21, wherein the NOS activity is characterized by one or more features selected from the group consisting of:

a) inducible by a plant pathogen;

b) having activity dependent on H_4B, FAD, FMN and NADPH;

c) requiring Ca^{2+} for activity;

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d) requiring calmodulin for activity; and

e) synthesizing NO as determined by a citrulline formation assay or by an oxy-hemoglobin assay.

23. The nucleic acid molecule of claim 22, wherein the NOS activity is further characterized by one or more features selected from the group consisting of:

a) activity inhibited by L-NMMA;

b) activity inhibited by aminoguanidine;

c) exhibiting calmodulin-mediated change in flavin fluorescence; and

d) exhibiting calmodulin-mediated change in tryptophan fluorescence.

24. The nucleic acid molecule of claim 21, isolated from tobacco or *Arabidopsis*.

25. The nucleic acid molecule of claim 21, wherein the enzyme has an amino acid sequence comprising one or more fragments selected from the group consisting of

FGVPMGYGGPHAAFLATSQEYKR (SEQ ID NO:2);

GNINIEEVRK (SEQ ID NO:3);

IAILNANYMAK (SEQ ID NO:4); and

GNADVQNNVL (SEQ ID NO:5).

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26. The nucleic acid molecule of claim 25, wherein the enzyme has amino acid SEQ ID NO:1.

27. The nucleic acid molecule of claim 26, having at least 90% sequence identity with SEQ ID NO:6.

28. The nucleic acid molecule of claim 26, having at least 60% sequence identity with a portion of SEQ NO:6 encoding an 80-residue amino-terminal portion of SEQ ID NO:1.

29. The nucleic acid molecule of claim 26, having a sequence that hybridizes with the complement of SEQ ID NO:6 under hybridization conditions comprising:

hybridization at least six hours at 42°C in 5X SSC, 5X Denhardt's reagent, 1.0% SDS, 100 μg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide; and

washing at least once at room temperature in 2X SSC and 0.1 - 1% SDS with a final wash at 65°C in 0.1X SSC and 0.1% SDS for 10 minutes.

30. The nucleic acid molecule of claim 26, having a sequence that hybridizes with the complement of a portion of SEQ ID NO:6 that encodes an 80-residue amino-terminal portion of SEQ ID NO:1, under hybridization conditions comprising:

hybridization at least six hours at 42°C in 5X SSC, 5X Denhardt's reagent,

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1.0% SDS, 100 μg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide; and

washing at least once at room temperature in 2X SSC and 0.1 - 1% SDS with a final wash at 45-55°C in 2X SSC and 0.1% SDS for10 minutes.

31. The nucleic acid molecule of claim 26, comprising SEQ ID NO:6.

32. The nucleic acid molecule of claim 21, which is a gene having an open reading frame that comprises the coding sequence.

33. A mRNA molecule produced by transcription of the gene of claim 32.

34. A cDNA molecule produced by reverse transcription of the mRNA molecule of claim 33.

35. An oligonucleotide between 8 and 100 bases in length, which is complementary to a segment of the nucleic acid molecule of claim 21.

36. A vector comprising the nucleic acid molecule of claim 21.

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37. The vector of claim 36, which is an expression vector selected from the group of vectors consisting of plasmid, cosmid, baculovirus, bacmid, bacterial, yeast and viral vectors.

38. The vector of claim 37, wherein the coding sequence of the nucleic acid molecule is operably linked to a constitutive promoter.

39. The vector of claim 37, wherein the coding sequence of the nucleic acid molecule is operably linked to an inducible promoter.

40. A host cell transformed with the vector of claim 37.

41. The host cell of claim 40, selected from the group consisting of plant cells, bacterial cells, fungal cells, insect cells and mammalian cells.

42. The host cell of claim 41, which is a plant cell selected from the group of plants consisting of tobacco, *Arabidopsis*, maize, wheat, rice, soybean barley, rye, oats, sorghum, alfalfa, clover, canola, safflower, sunflower, peanut, tomato tomatillo, potato, pepper, eggplant, sugar beet, carrot, cucumber, lettuce, pea, aster, begonia, chrysanthemum, delphinium, zinnia, and turfgrasses.

43. A fertile plant produced from the plant cell of claim 42.

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44. A method for identifying agents that modulate piNOS enzymatic activity, comprising the steps of:

a) combining a pre-determined amount of the piNOS with a substrate acted upon by the piNOS to produce a product, wherein the substrate or the product are directly or indirectly detectable, the combining being performed in the presence or absence of a test compound suspected of modulating the piNOS activity; and

b) measuring conversion of the substrate to the product in the presence and absence of the test compound, a change in rate or total amount of conversion of the substrate to the product in the presence of the test compound, as compared to in the absence of the test compound, being indicative that the test compound modulates piNOS enzymatic activity.

45. The method of claim 44, wherein the piNOS is affixed to a solid support.

46. The method of claim 44, adapted for identifying agents that modulate piNOS enzymatic activity in host cells, comprising the steps of:

a) introducing a piNOS-encoding nucleic acid molecule into the host cells under conditions whereby piNOS is produced by expression of the nucleic acid molecule;

b) treating a sample of the host cells with a test compound suspected of modulating the piNOS enzymatic activity; and

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c) comparing piNOS enzymatic activity in the sample of host cells treated with the test compound with piNOS enzymatic activity in an equivalent sample of host cells not treated with the test compound, a difference between the comparative piNOS enzymatic activities being indicative that the test compound modulates piNOS enzymatic activity.

47. A method for identifying agents that modulate expression of genes encoding piNOS in host cells, comprising the steps of:

a) introducing into the host cells a piNOS-encoding gene comprising a *piNOS* coding region and *piNOS* expression regulatory sequences;

b) treating a sample of the host cells with a test compound suspected of modulating genes encoding piNOS; and

c) comparing piNOS-encoding gene expression in the sample of host cells treated with the test compound with piNOS-encoding gene expression in an equivalent sample of host cells not treated with the test compound, a difference between the comparative piNOS-encoding gene expressions being indicative that the test compound modulates expression of genes encoding piNOS.

48. The method of claim 47, wherein the piNOS-encoding gene expression is measured by measuring piNOS-encoding mRNA.

49. The method of claim 47, wherein the piNOS-encoding gene expression is measured by measuring piNOS protein.

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50. The method of claim 47, wherein the piNOS-encoding gene expression is measured by measuring piNOS enzymatic activity.

51. The method of claim 47, wherein the coding sequence is replaced by a heterologous coding sequence encoding a detectable gene product, and the piNOS-encoding gene expression is measured by measuring the detectable gene product.

52. A method to enhance resistance of a plant to plant pathogens or other disease causing agents, comprising increasing an amount or activity in the plant of a piNOS enzyme having nitric oxide synthase (NOS) activity, wherein the NOS activity is inducible in a plant and comprises production of nitric oxide from arginine.

53. The method of claim 52, comprising over-expressing a gene encoding the piNOS enzyme in the plant.

54. The method of claim 53, comprising introducing a piNOS-encoding transgene into the plant.

55. The method of claim 52, wherein the piNOS activity is enhanced by treating the plant with a modulator of piNOS enzymatic activity identified by the method of claim 44, of a type that increases piNOS enzymatic activity.

56. The method of claim 53, wherein the piNOS-encoding gene expression is further enhanced by treating the plant with a modulator of piNOS-encoding gene

expression identified by the method of claim 47, of a type that increases expression of genes encoding piNOS.

57. A method of inhibiting expression of a piNOS-encoding gene in a plant, the method comprising introducing a nucleic acid molecule into the plant that inhibits the expression of the plant's piNOS-encoding gene.

58. The method of claim 57, wherein the nucleic acid encodes a molecule that is antisense to SEQ ID NO:6.

59. The method of claim 57, wherein the nucleic acid comprises a double stranded RNA segment of SEQ ID NO:6.

60. The method of claim 57, wherein the nucleic acid comprises a piNOS-encoding sequence and wherein the inhibition of expression the plant's piNOS-encoding gene occurs through co-suppression by the introduced nucleic acid.

61. A method to inhibit piNOS-mediated signal transduction in a plant, the method comprising introducing into the plant a mutated piNOS-encoding nucleic acid, which encodes a piNOS protein lacking NOS activity, wherein the NOS activity is inducible in a plant and comprises production of nitric oxide from arginine.

62. A transgenic plant having enhanced resistance to plant pathogens or other disease-causing agents, comprising a transgene that encodes a plant enzyme having

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nitric oxide synthase (NOS) activity, wherein the NOS activity is inducible in the plant and comprises production of nitric oxide from arginine.

63. The transgenic plant of claim 62, wherein the transgene encodes SEQ ID NO:1.

64. The transgenic plant of claim 62, which is fertile.

65. A transgenic knock-out plant, wherein expression or activity of the plant's endogenous enzyme having a nitric oxide synthase (NOS) activity that is inducible in the plant and comprises production of nitric oxide from arginine is absent or reduced as compared to a non-transgenic plant of the same type.

66. The transgenic knock-out plant of claim 65, which is fertile.

67. A kit for producing transgenic host cells that produce a piNOS enzyme, which comprises the vector of claim 36 and instructions for producing the transgenic cells using the vector.

68. The kit of claim 67, which optionally comprises one or more additional components selected from the group consisting of:

a) culture media for culturing the cells;

b) reagents for performing transformation of the cells;

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c) reagents for testing the transgenic cells for piNOS-encoding gene expression; and

d) reagents for testing the transgenic cells for piNOS enzymatic activity.

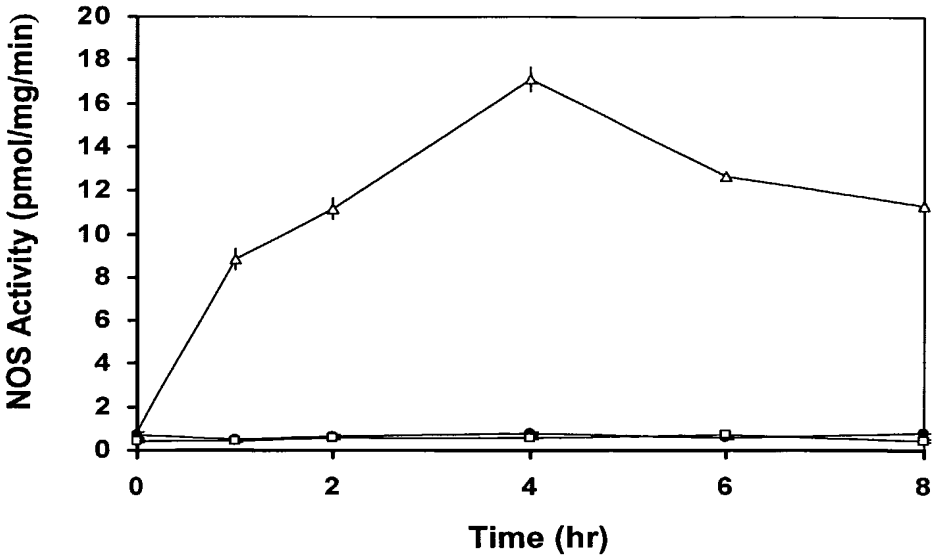

NOS Activity (pmol/mg/min)
20
18
16
14
12
10
8
6
4
2
0
0
2
4
6
8
Time (hr)

FIG. 1A

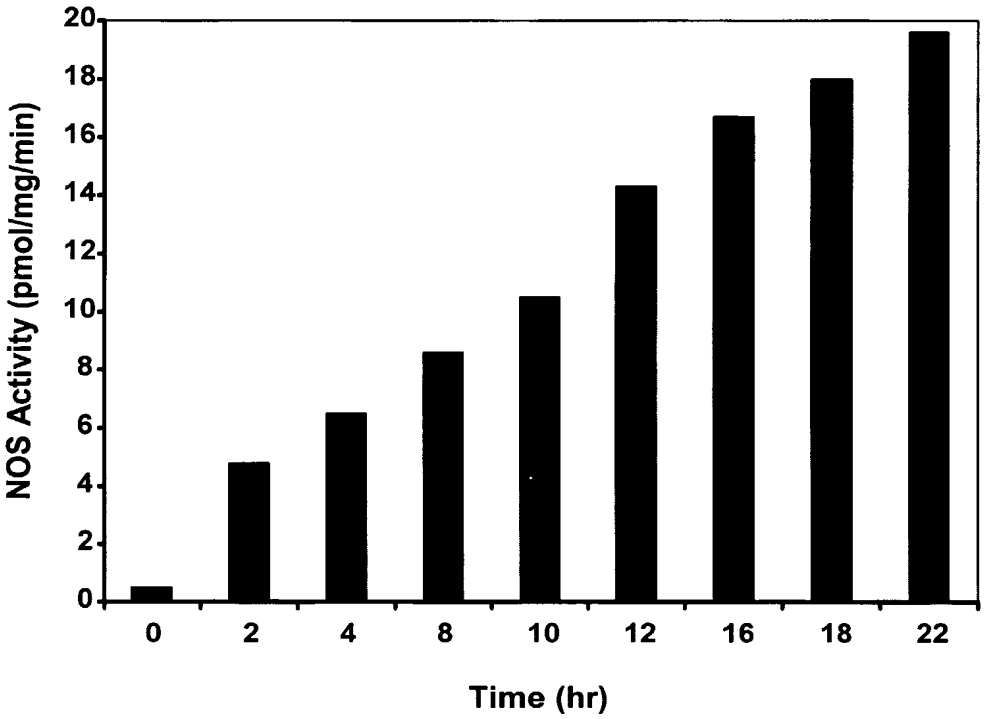

NOS Activity (pmol/mg/min)
20
18
16
14
12
10
8
6
4
2
0
0
2
4
8
10
12
16
18
22
Time (hr)

FIG. 1B

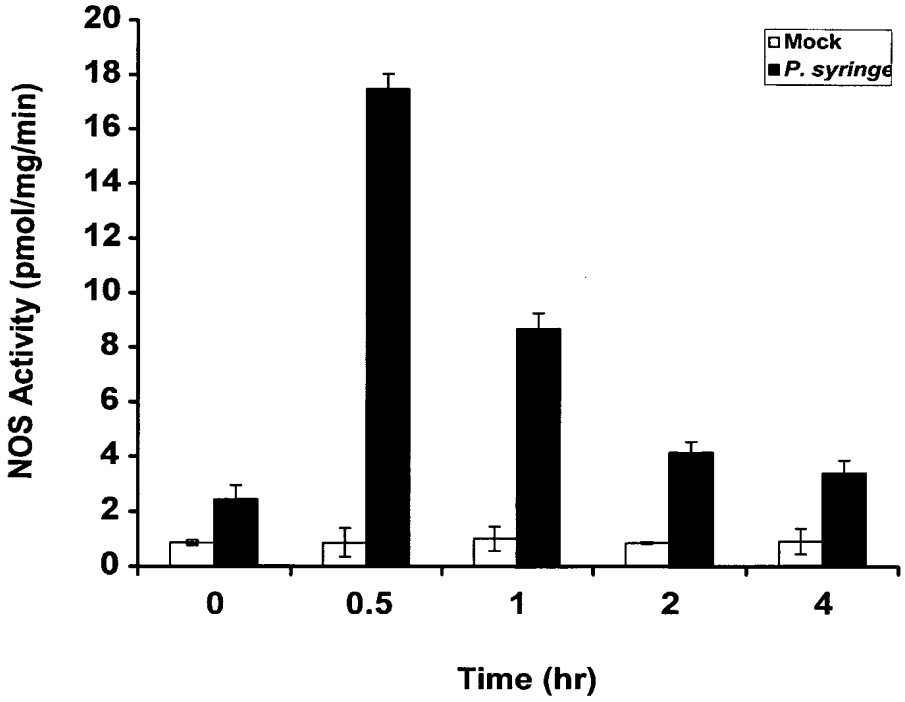

NOS Activity (pmol/mg/min)
20
18
16
14
12
10
8
6
4
2
0
0
0.5
1
2
4
Time (hr)
Mock
P. syringe

FIG. 2

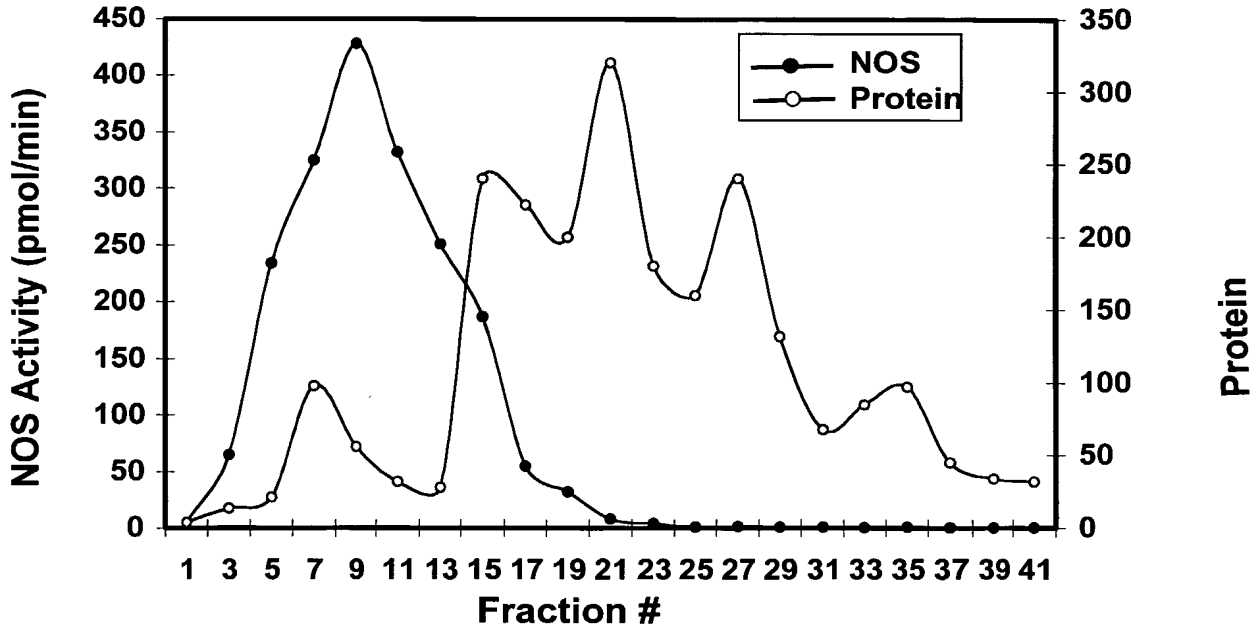

NOS
Protein
NOS Activity (pmol/min)
Protein
Fraction #
450
400
350
300
250
200
150
100
50
0
350
300
250
200
150
100
50
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41

FIG. 3

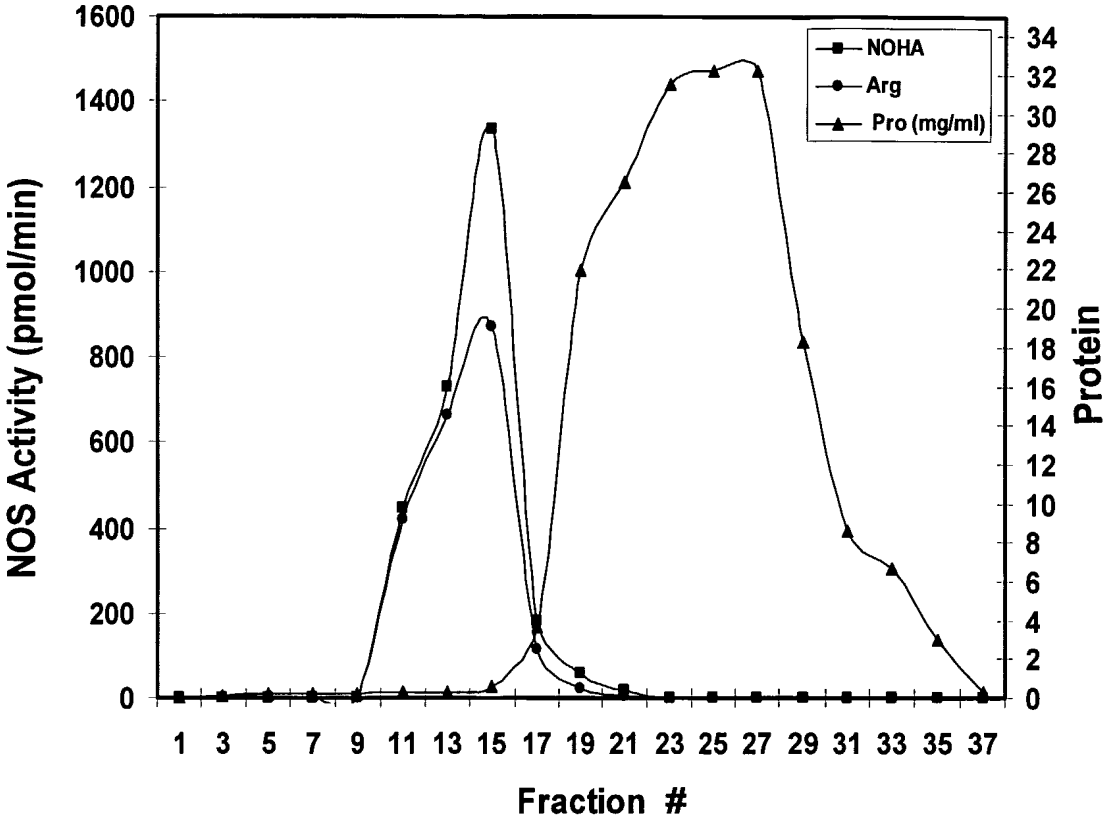

NOS Activity (pmol/min)
Protein
Fraction #
NOHA
Arg
Pro (mg/ml)
1600
1400
1200
1000
800
600
400
200
0
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37

FIG. 4

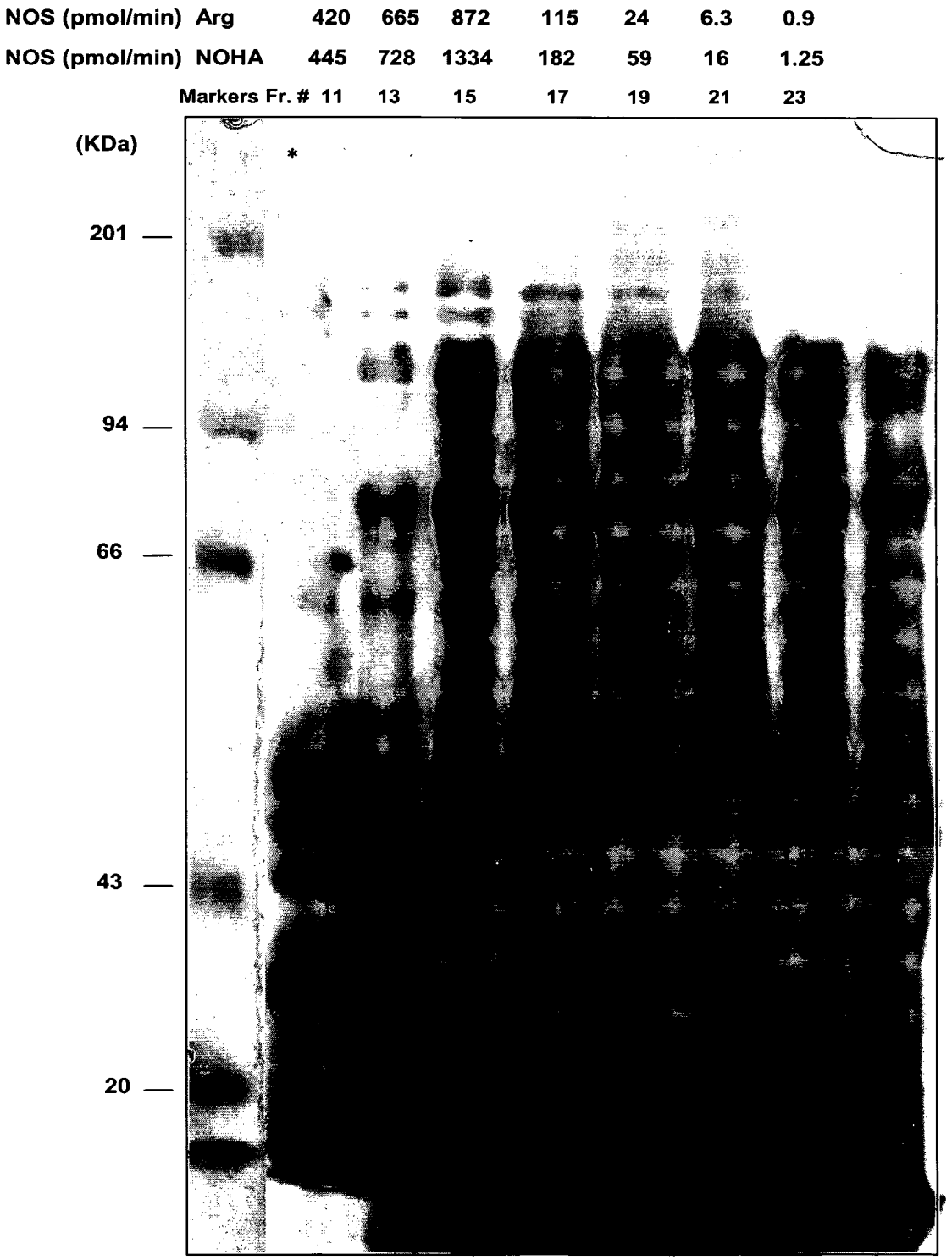

NOS (pmol/min) Arg 420 665 872 115 24 6.3 0.9
NOS (pmol/min) NOHA 445 728 1334 182 59 16 1.25
Markers Fr. # 11 13 15 17 19 21 23
(KDa)
201
94
66
43
20
*

FIG. 5

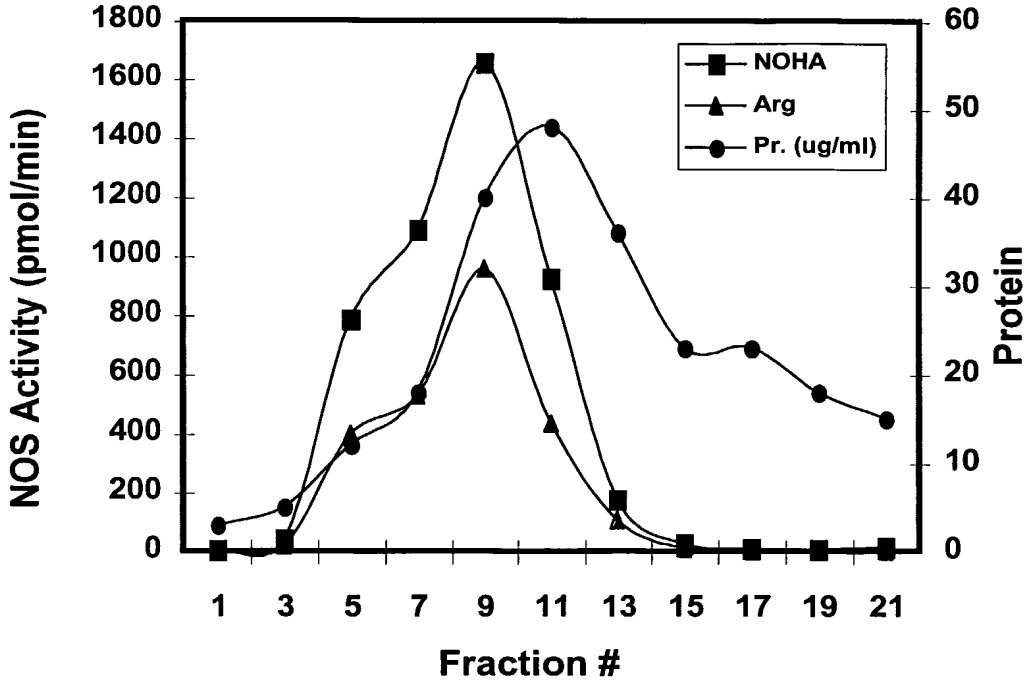

NOS Activity (pmol/min)
1800
1600
1400
1200
1000
800
600
400
200
0
60
50
40
30
20
10
0
Protein
NOHA
Arg
Pr. (ug/ml)
1 3 5 7 9 11 13 15 17 19 21
Fraction #

FIG. 6

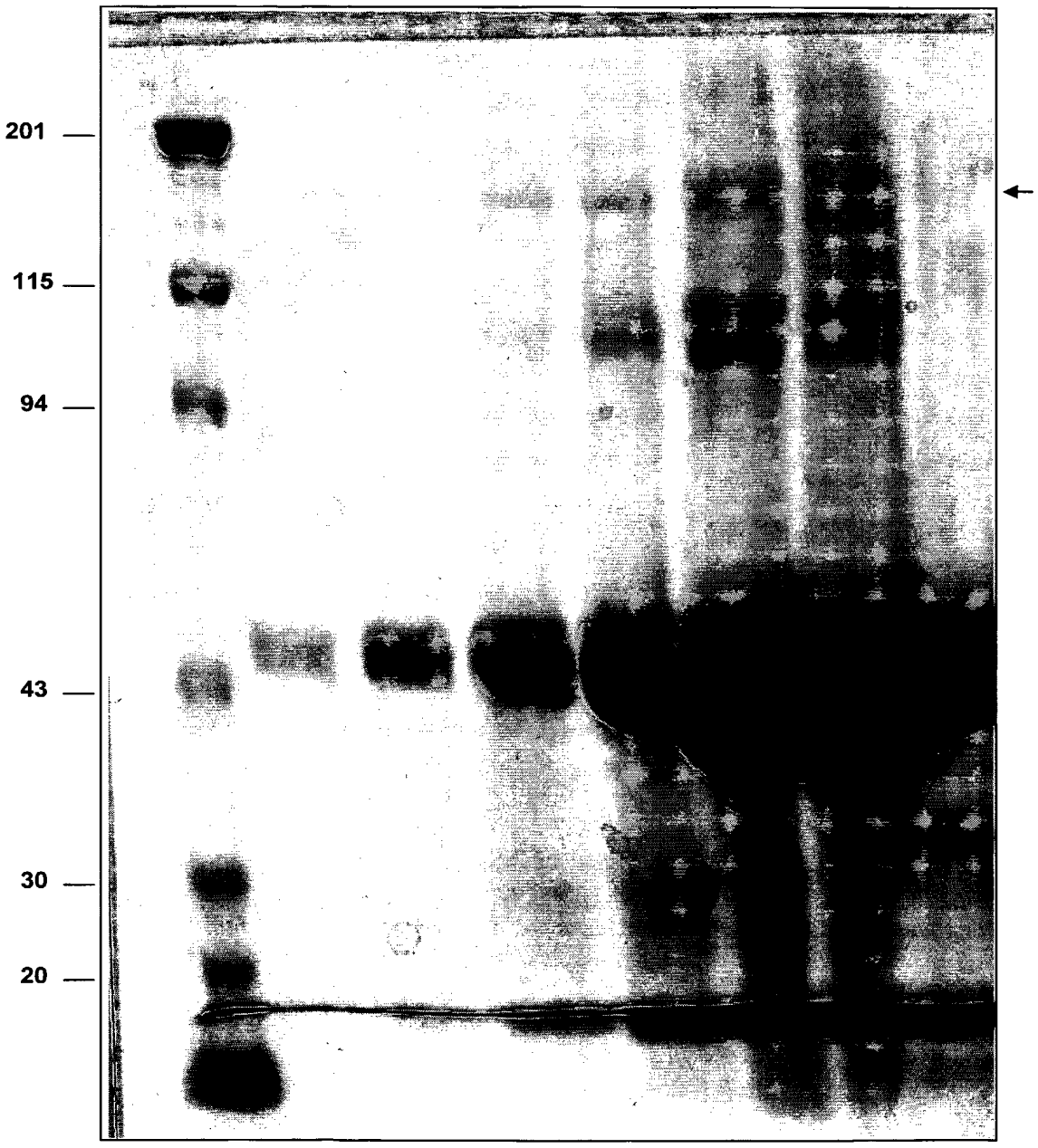

NOS (pmol/min) Arg 1 18 397 527 956 435 105
NOS (pmol/min) NOHA 3 41 785 1089 1656 923 176
(KDa) Markers Fr.# 1 3 5 7 9 11 13
201
115
94
43
30
20

FIG. 7

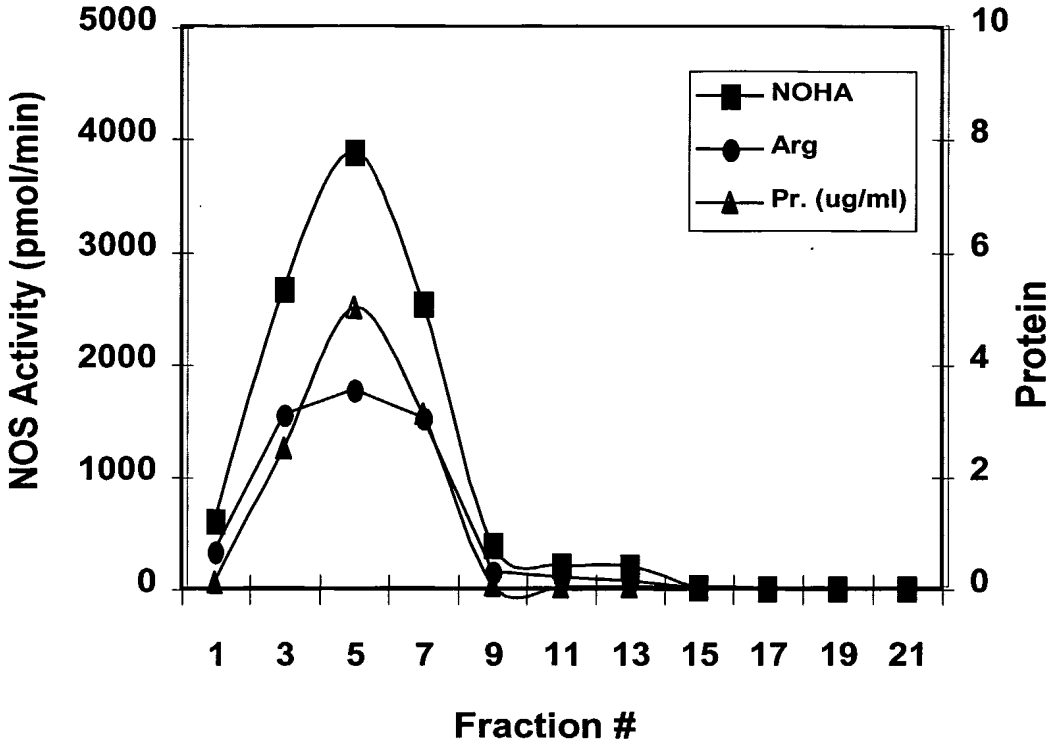

5000
4000
3000
2000
1000
0
NOS Activity (pmol/min)
10
8
6
4
2
0
Protein
NOHA
Arg
Pr. (ug/ml)
1 3 5 7 9 11 13 15 17 19 21
Fraction #

FIG. 8

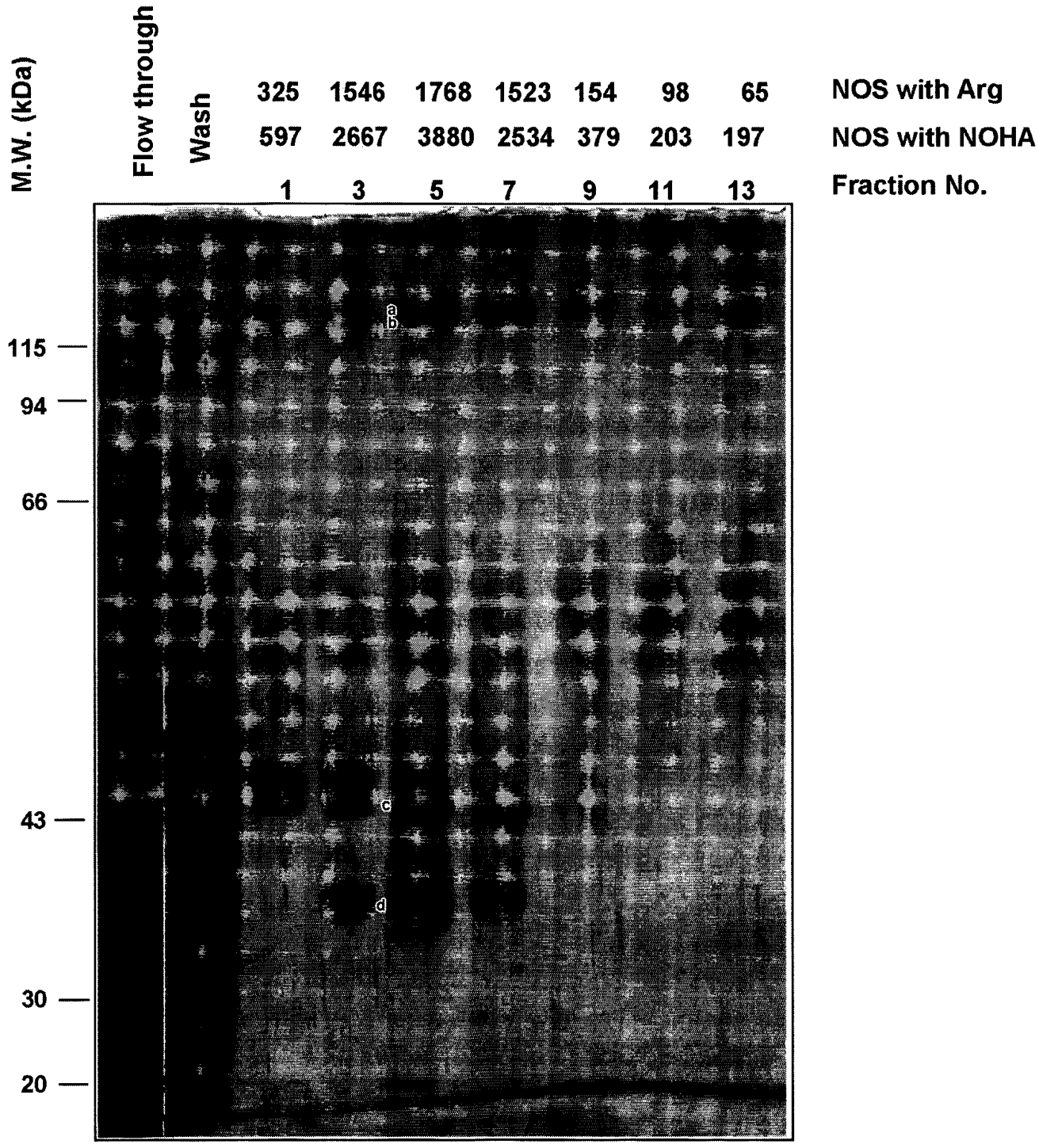

M.W. (kDa)
Flow through
Wash
325 1546 1768 1523 154 98 65
NOS with Arg
597 2667 3880 2534 379 203 197
NOS with NOHA
1 3 5 7 9 11 13
Fraction No.
115
94
66
43
30
20
a
b
c
d

FIG. 9

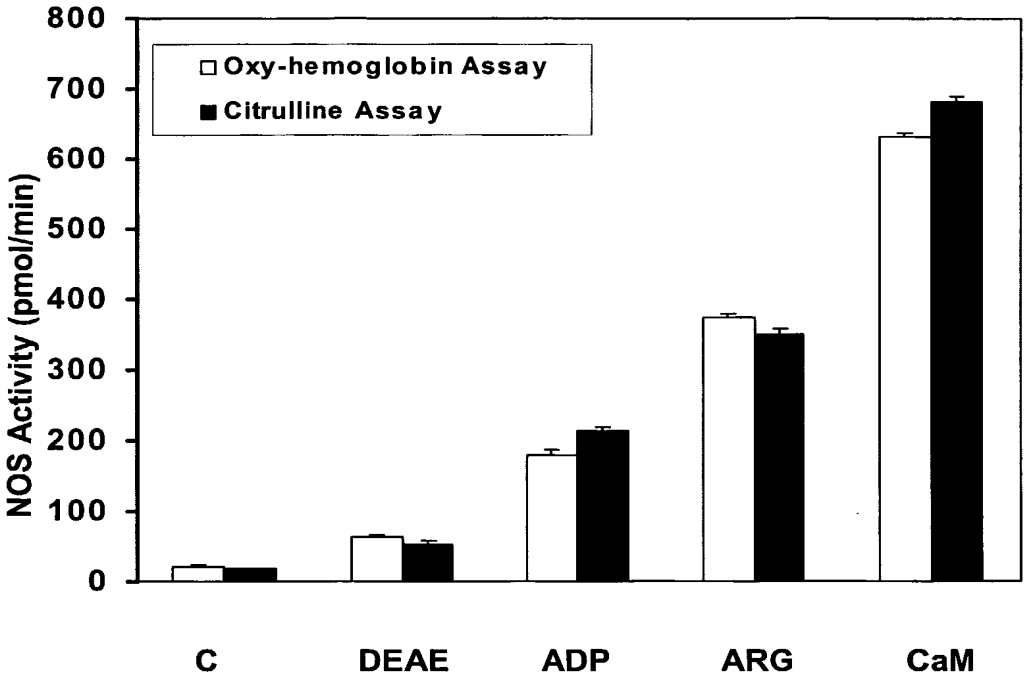

800
700
600
500
400
300
200
100
0
NOS Activity (pmol/min)
Oxy-hemoglobin Assay
Citrulline Assay
C
DEAE
ADP
ARG
CaM

FIG. 10

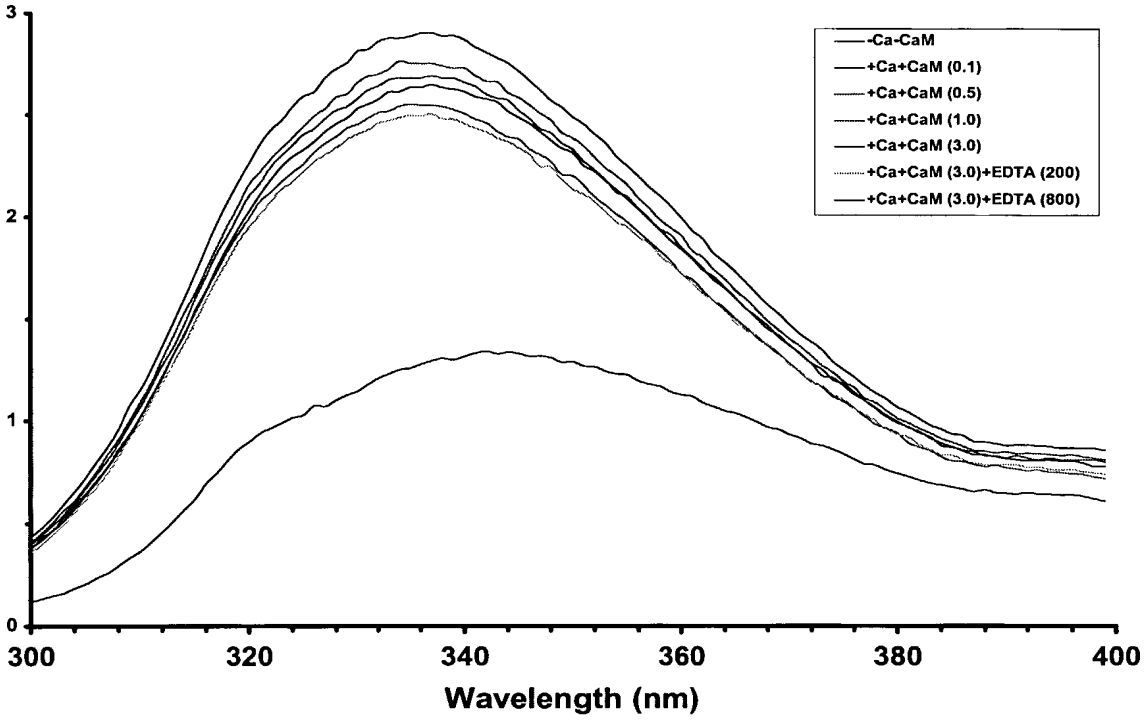

-Ca-CaM
+Ca+CaM (0.1)
+Ca+CaM (0.5)
+Ca+CaM (1.0)
+Ca+CaM (3.0)
+Ca+CaM (3.0)+EDTA (200)
+Ca+CaM (3.0)+EDTA (800)
3
2
1
0
300
320
340
360
380
400
Wavelength (nm)

FIG. 12

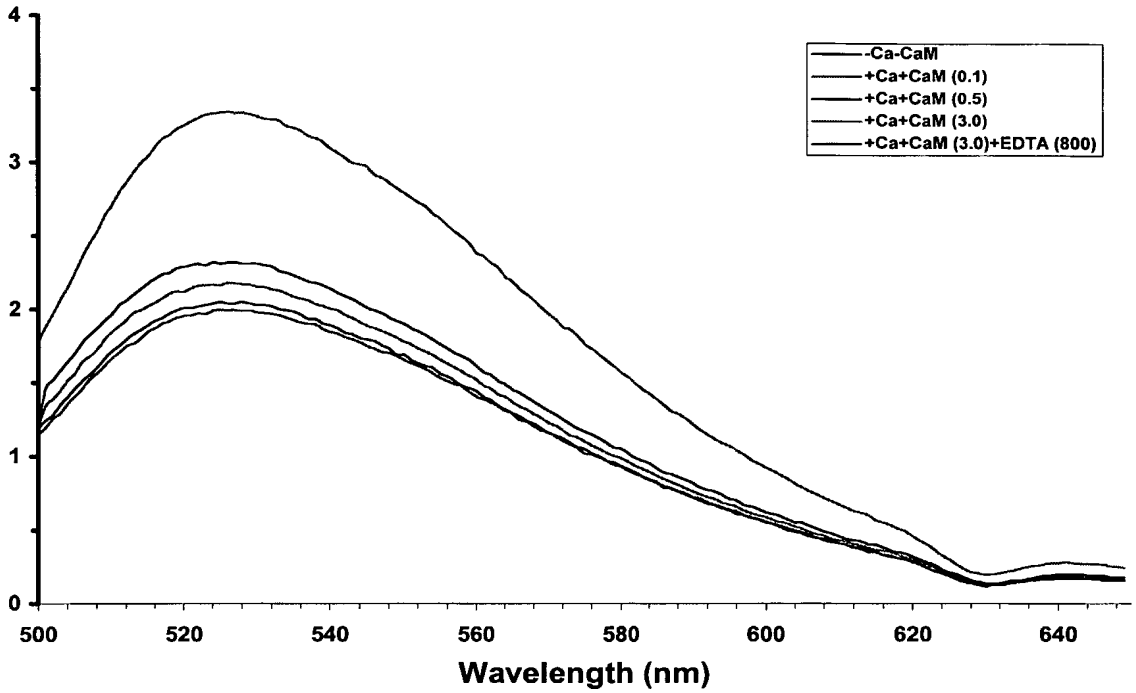

-Ca-CaM
+Ca+CaM (0.1)
+Ca+CaM (0.5)
+Ca+CaM (3.0)
+Ca+CaM (3.0)+EDTA (800)
4
3
2
1
0
500
520
540
560
580
600
620
640
Wavelength (nm)

FIG. 13

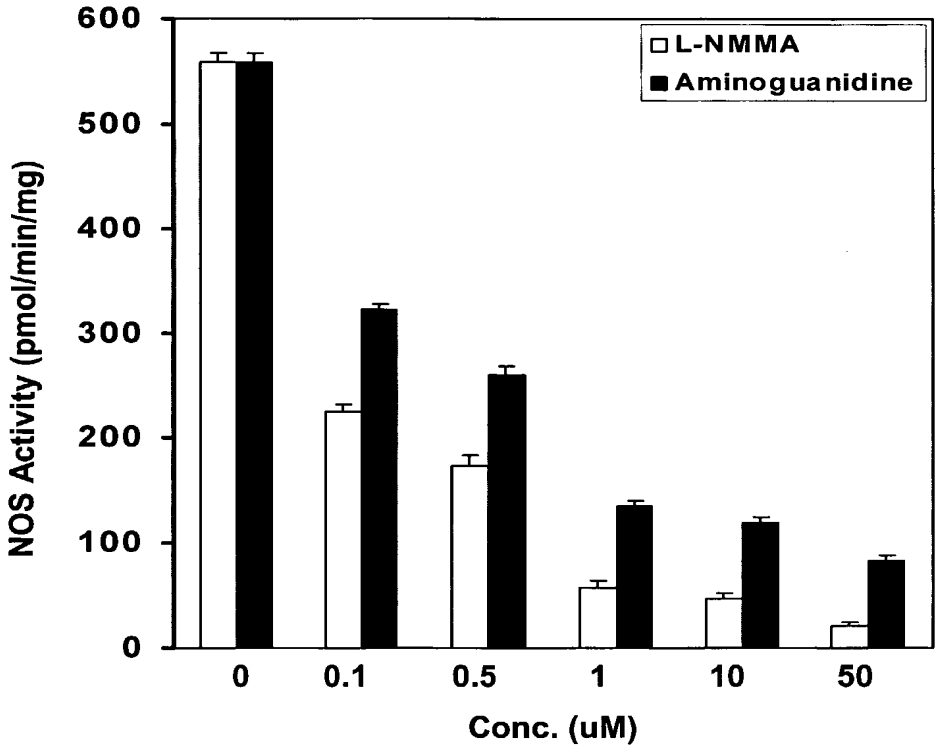

600
500
400
300
200
100
0
NOS Activity (pmol/min/mg)
L-NMMA
Aminoguanidine
0
0.1
0.5
1
10
50
Conc. (uM)

FIG. 14

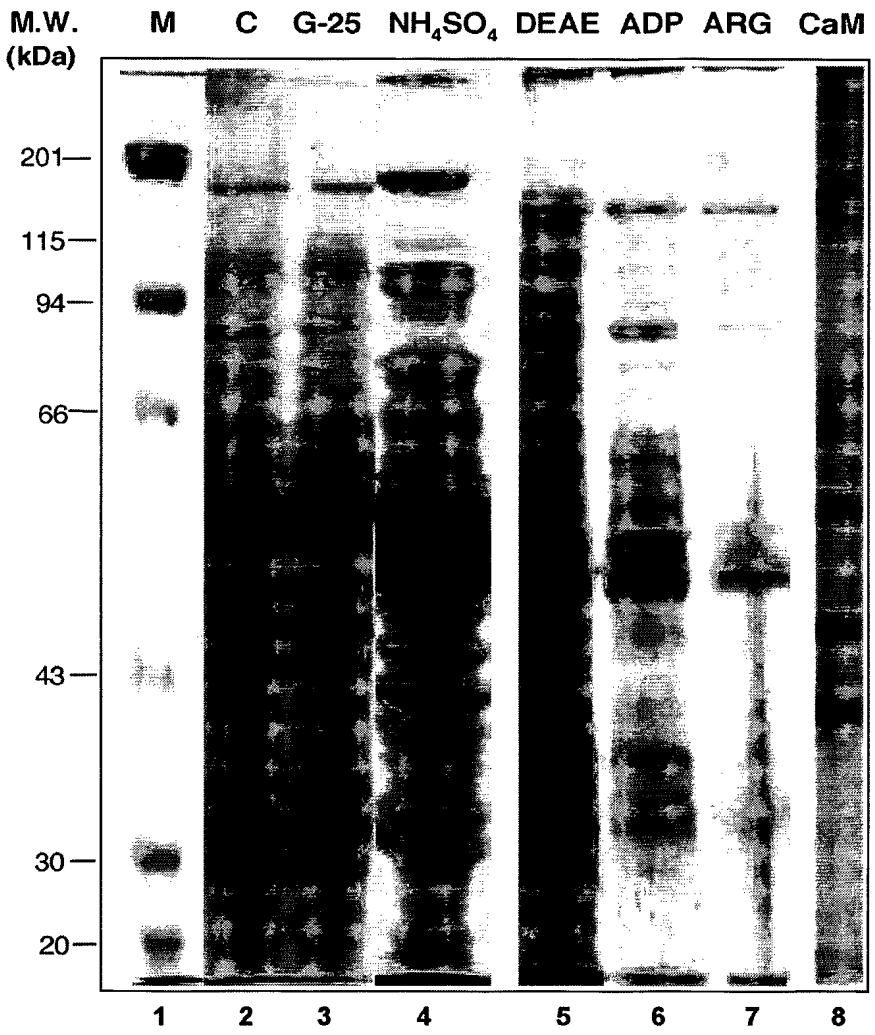
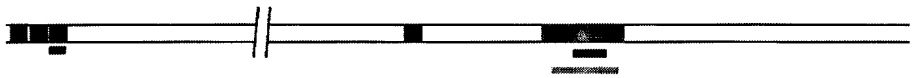

M.W.
(kDa)
M
C
G-25
NH4SO4
DEAE
ADP
ARG
CaM
201
115
94
66
43
30
20
1
2
3
4
5
6
7
8

FIG. 11

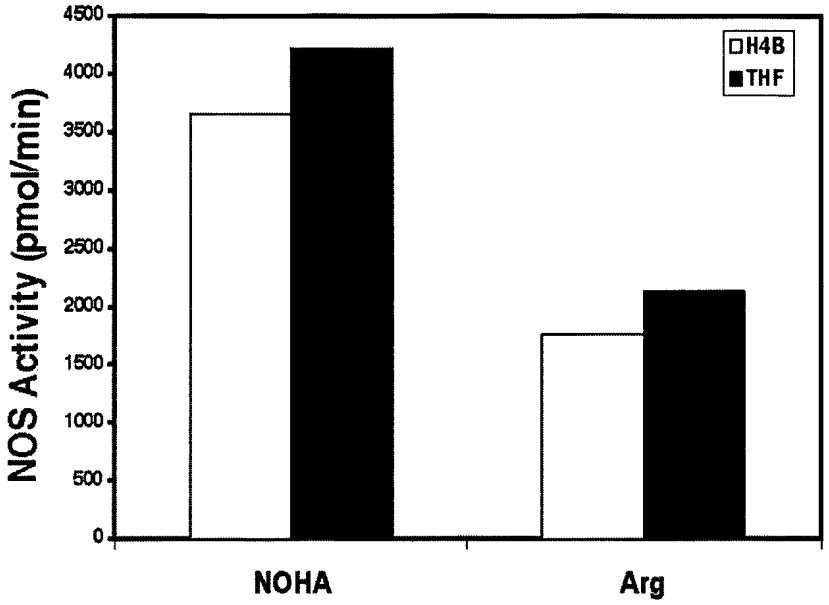
```
MERARRLAYRGIVKRLVNDTKRHRNAETPHLVPHAPARYVSSLSPFISTPRSVNHTAAFGRHQQTRSISVDAVKPSDTFP    80
RRHNSATPDEQTHMAKFCGFDHIDSLIDATVPKSIRLDSMKFSKFDAGLTESQMIQHMVDLASKNKVFKSFIGMGYYNTH   160
VPTVILRNIMENPAWYTQYTPYQAEISQGRLESLLNFQTVITDLTGLPMSNASLLDEGTAAAEAMAMCNNILKGKKKTFV   240
IASNCHPQTIDVCKTRADGFDLKVVTSDLKDIDYSSGDVCGVLVQYPGTEGEVLDYAEFVKNAHANGVKVVMATDLLAL   320
TVLKPPGEFGADIVVGSAQRFGVPMGYGGPHAAFLATSQEYKRMMPGRIIGISVDSSGKQALRMAMQTREQHIRRDKATS   400
NICTAQALLANMAAMYAVYHGPAGLKSIAQRVHGLAGIFSLGLNKLGVAEVQELPFFDTVKIKCSDAHAIADAASKSEIN   480
LRVVDSTTITASFDETTTLDDVDKLFKVFASGKPVPFTAESLAPEVQNSIPSSLTRESPYLTHPIFNMYHTEHELLRYIHKL   560
QSKDLSLCHSMIPLGSCTMKLNATTEMMPVTWPSFTDIHPFAPVEQAQGYQEMFENLGDLLCTITGFDSFSLQPNAGAA   640
GEYAGLMVIRAYHMSRGDHHRNVCIIPVSAHGTNPASAAMCGMKIITVGTDAKGNINIEEVRKAAEANKDNLAALMVTY   720
PSTHGVYEEGIDEICNIIHENGGQVYMDGANMNAQVGLTSPGFIGADVCHLNLHKTFCIPHGGGGPGMGPIGVKNHLAPFL   800
PSHPVIPTGGIPQPEKTAPLGAISAAPWGSALILPISYTYIAMMGSGGLTDASKIAILNANYMAKRLEKHYPVLFRGVNGTV   880
AREFIIDLRGFKNTAGIEPEDVAKRLMDYGFHGPTMSWPVPGTLMIEPTESESKAELDRFCDALISIREEIAQIEKGNADVQN   960
NVLKGAPHPPSLLMADTWKKPYSREYAAFPAPWLRSSKFWPTTGRVDNVYGDRKLVCTLLPEEEQVAAAVSA          1037
```

FIG. 15A



- CaM binding site found in NOSs (8-28 aa)
- Heme-oxygenase active site, functionally similar to the oxygenase domain of NOSs (32-44 aa)
- FAD/FMN binding site found in NOSs (33-58 aa)
- NADPH-dependent cytochrome P450 reductase domain similar to that found in NOSs (33-52 aa)
- Leucine zipper, possibly for dimerization (575-596 aa)
- Pyridoxal phosphate binding domain (746-813 aa)
- Potential heme attachment site
- NADPH binding-Rossmann fold domain, it can also bind tetrahydrofolate, which can replace tetrahydrobiopterin (H_4B) used by NOSs

FIG. 15B


NOS Activity (pmol/min)
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4000
3500
3000
2500
2000
1500
1000
500
0
H4B
THF
NOHA
Arg

FIG. 16

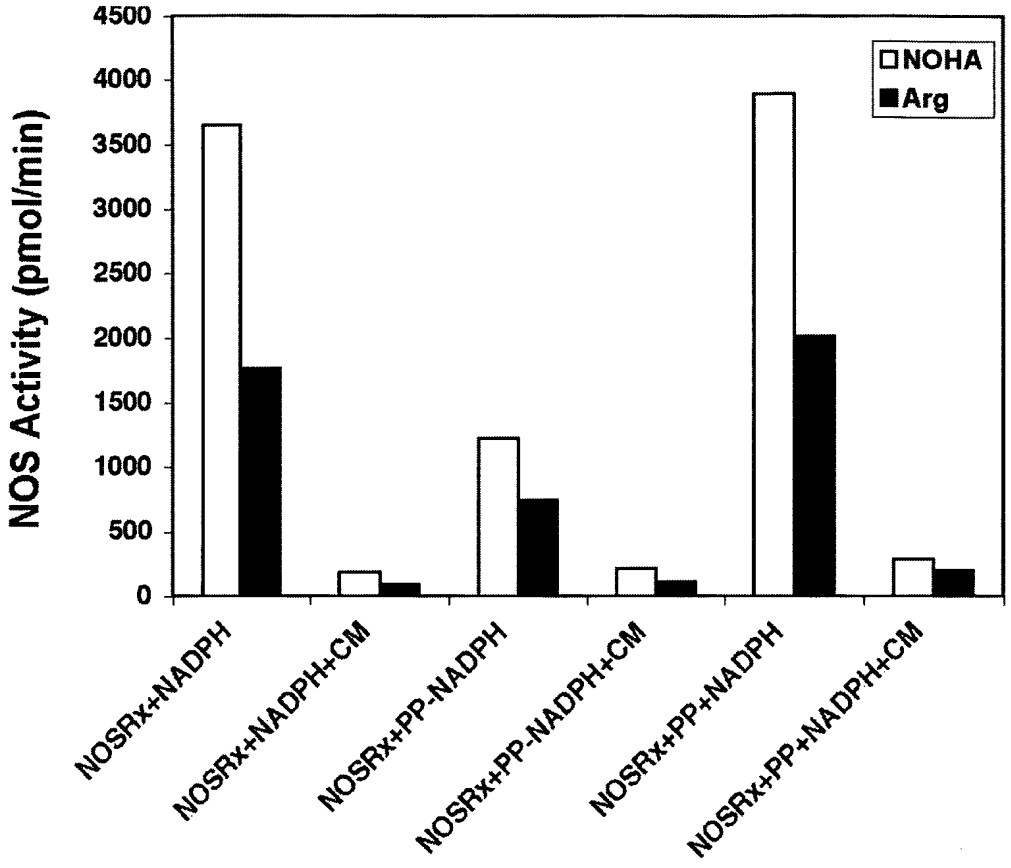

NOS Activity (pmol/min)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
NOHA
Arg
NOSRx+NADPH
NOSRx+NADPH+CM
NOSRx+PP-NADPH
NOSRx+PP-NADPH+CM
NOSRx+PP+NADPH
NOSRx+PP+NADPH+CM

FIG. 17

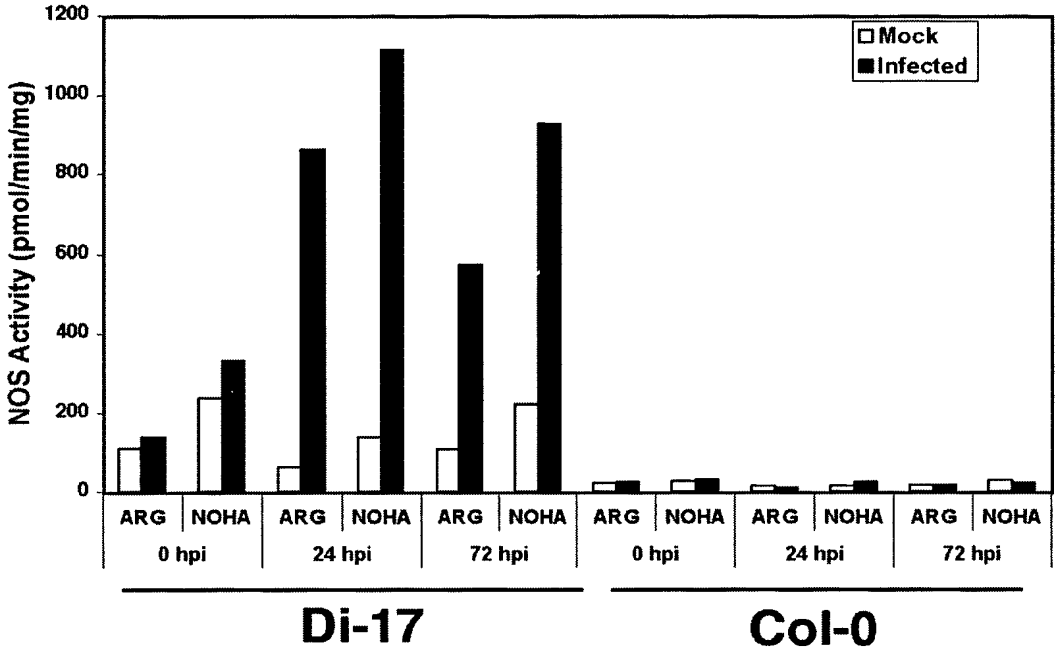
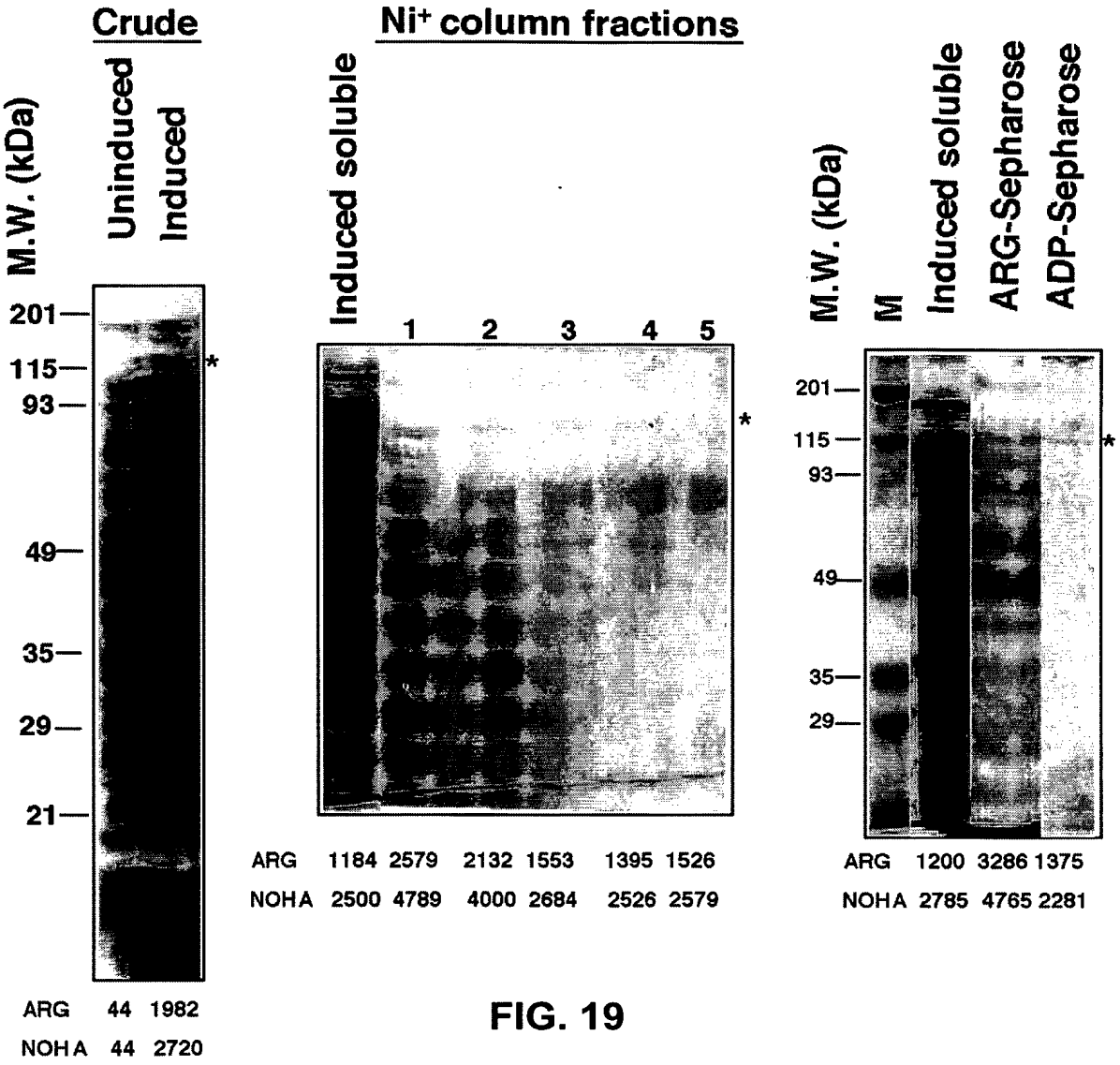

1200
1000
800
600
400
200
0
NOS Activity (pmol/min/mg)
Mock
Infected
ARG
NOHA
0 hpi
24 hpi
72 hpi
Di-17
Col-0

FIG. 18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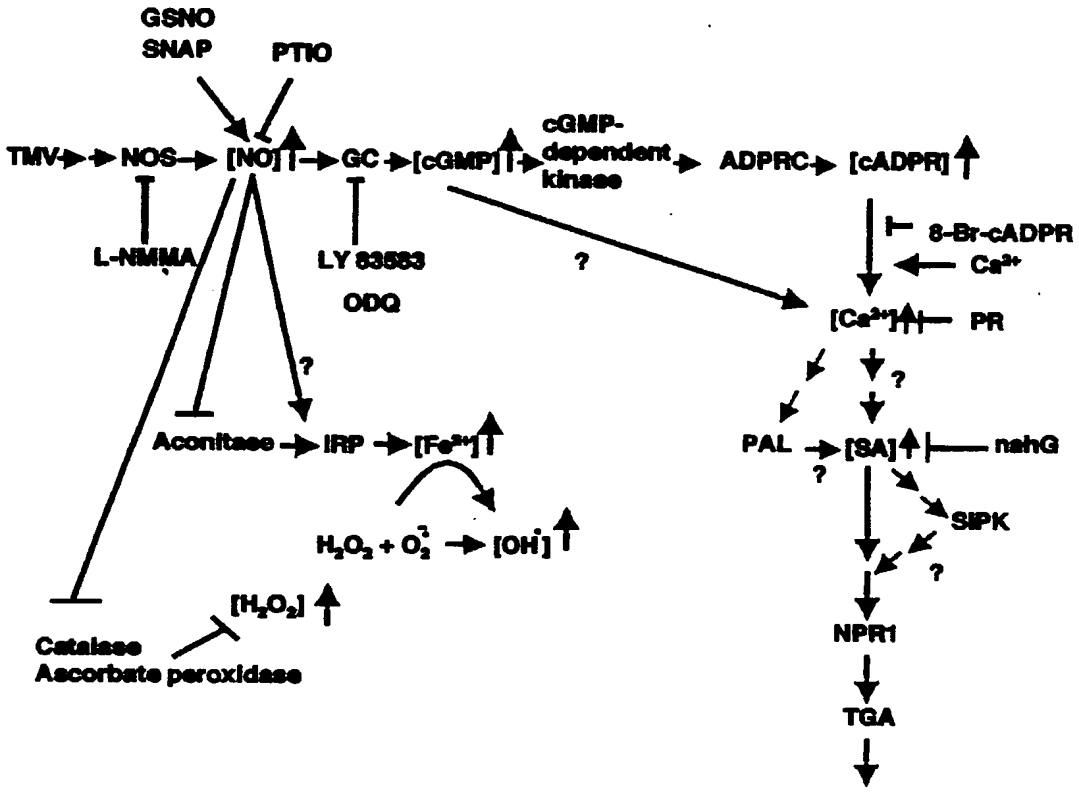
M.W. (kDa)
Uninduced
Induced
201
115
93
49
35
29
21
*
ARG 44 1982
NOHA 44 2720
Ni+ column fractions
Induced soluble
1 2 3 4 5
*
ARG 1184 2579 2132 1553 1395 1526
NOHA 2500 4789 4000 2684 2526 2579
M.W. (kDa)
M
Induced soluble
ARG-Sepharose
ADP-Sepharose
201
115
93
49
35
29
*
ARG 1200 3286 1375
NOHA 2785 4765 2281

FIG. 19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PTIO
TMV
NOS
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GC
[cGMP]
cGMP-dependent kinase
ADPRC
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8-Br-cADPR
Ca^{2+}
L-NMMA
LY 83583
ODQ
?
$[Ca^{2+}]$
PR
Aconitase
IRP
$[Fe^{2+}]$
PAL
[SA]
nahG
SIPK
$H_2O_2 + O_2^-$
$[OH^-]$
$[H_2O_2]$
Catalase
Ascorbate peroxidase
NPR1
TGA

FIG. 20

SEQUENCE LISTING

<110> Boyce Thompson Institute for Plant Reseach
Klessig, Daniel F.
Chandok, Meena

<120> Plant Nitric Oxide Synthase

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<150> 60/363,239
<151> 2002-03-11

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35 40 45

Thr Pro Arg Ser Val Asn His Thr Ala Ala Phe Gly Arg His Gln Gln
50 55 60

Thr Arg Ser Ile Ser Val Asp Ala Val Lys Pro Ser Asp Thr Phe Pro
65 70 75 80

Arg Arg His Asn Ser Ala Thr Pro Asp Glu Gln Thr His Met Ala Lys
85 90 95

Phe Cys Gly Phe Asp His Ile Asp Ser Leu Ile Asp Ala Thr Val Pro
100 105 110

Lys Ser Ile Arg Leu Asp Ser Met Lys Phe Ser Lys Phe Asp Ala Gly
115 120 125

Leu Thr Glu Ser Gln Met Ile Gln His Met Val Asp Leu Ala Ser Lys
130 135 140

Asn Lys Val Phe Lys Ser Phe Ile Gly Met Gly Tyr Tyr Asn Thr His
145 150 155 160

Val Pro Thr Val Ile Leu Arg Asn Ile Met Glu Asn Pro Ala Trp Tyr
165 170 175

Thr Gln Tyr Thr Pro Tyr Gln Ala Glu Ile Ser Gln Gly Arg Leu Glu
180 185 190

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Ser Leu Leu Asn Phe Gln Thr Val Ile Thr Asp Leu Thr Gly Leu Pro
        195                 200                 205

Met Ser Asn Ala Ser Leu Leu Asp Glu Gly Thr Ala Ala Ala Glu Ala
    210                 215                 220

Met Ala Met Cys Asn Asn Ile Leu Lys Gly Lys Lys Lys Thr Phe Val
225                 230                 235                 240

Ile Ala Ser Asn Cys His Pro Gln Thr Ile Asp Val Cys Lys Thr Arg
                245                 250                 255

Ala Asp Gly Phe Asp Leu Lys Val Val Thr Ser Asp Leu Lys Asp Ile
            260                 265                 270

Asp Tyr Ser Ser Gly Asp Val Cys Gly Val Leu Val Gln Tyr Pro Gly
        275                 280                 285

Thr Glu Gly Glu Val Leu Asp Tyr Ala Glu Phe Val Lys Asn Ala His
    290                 295                 300

Ala Asn Gly Val Lys Val Val Met Ala Thr Asp Leu Leu Ala Leu Thr
305                 310                 315                 320

Val Leu Lys Pro Pro Gly Glu Phe Gly Ala Asp Ile Val Val Gly Ser
                325                 330                 335

Ala Gln Arg Phe Gly Val Pro Met Gly Tyr Gly Gly Pro His Ala Ala
            340                 345                 350

Phe Leu Ala Thr Ser Gln Glu Tyr Lys Arg Met Met Pro Gly Arg Ile
        355                 360                 365

Ile Gly Ile Ser Val Asp Ser Ser Gly Lys Gln Ala Leu Arg Met Ala
    370                 375                 380

Met Gln Thr Arg Glu Gln His Ile Arg Arg Asp Lys Ala Thr Ser Asn
385                 390                 395                 400

Ile Cys Thr Ala Gln Ala Leu Leu Ala Asn Met Ala Ala Met Tyr Ala
                405                 410                 415

Val Tyr His Gly Pro Ala Gly Leu Lys Ser Ile Ala Gln Arg Val His
            420                 425                 430

Gly Leu Ala Gly Ile Phe Ser Leu Gly Leu Asn Lys Leu Gly Val Ala
        435                 440                 445

Glu Val Gln Glu Leu Pro Phe Phe Asp Thr Val Lys Ile Lys Cys Ser
    450                 455                 460

Asp Ala His Ala Ile Ala Asp Ala Ala Ser Lys Ser Glu Ile Asn Leu
465                 470                 475                 480
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Arg Val Val Asp Ser Thr Thr Ile Thr Ala Ser Phe Asp Glu Thr Thr
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Thr Leu Asp Asp Val Asp Lys Leu Phe Lys Val Phe Ala Ser Gly Lys
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Pro Val Pro Phe Thr Ala Glu Ser Leu Ala Pro Glu Val Gln Asn Ser
        515                 520                 525

Ile Pro Ser Ser Leu Thr Arg Glu Ser Pro Tyr Leu Thr His Pro Ile
    530                 535                 540

Phe Asn Met Tyr His Thr Glu His Glu Leu Leu Arg Tyr Ile His Lys
545                 550                 555                 560

Leu Gln Ser Lys Asp Leu Ser Leu Cys His Ser Met Ile Pro Leu Gly
                565                 570                 575

Ser Cys Thr Met Lys Leu Asn Ala Thr Thr Glu Met Met Pro Val Thr
            580                 585                 590

Trp Pro Ser Phe Thr Asp Ile His Pro Phe Ala Pro Val Glu Gln Ala
        595                 600                 605

Gln Gly Tyr Gln Glu Met Phe Glu Asn Leu Gly Asp Leu Leu Cys Thr
    610                 615                 620

Ile Thr Gly Phe Asp Ser Phe Ser Leu Gln Pro Asn Ala Gly Ala Ala
625                 630                 635                 640

Gly Glu Tyr Ala Gly Leu Met Val Ile Arg Ala Tyr His Met Ser Arg
                645                 650                 655

Gly Asp His His Arg Asn Val Cys Ile Ile Pro Val Ser Ala His Gly
            660                 665                 670

Thr Asn Pro Ala Ser Ala Ala Met Cys Gly Met Lys Ile Ile Thr Val
        675                 680                 685

Gly Thr Asp Ala Lys Gly Asn Ile Asn Ile Glu Glu Val Arg Lys Ala
    690                 695                 700

Ala Glu Ala Asn Lys Asp Asn Leu Ala Ala Leu Met Val Thr Tyr Pro
705                 710                 715                 720

Ser Thr His Gly Val Tyr Glu Glu Gly Ile Asp Glu Ile Cys Asn Ile
                725                 730                 735

Ile His Glu Asn Gly Gly Gln Val Tyr Met Asp Gly Ala Asn Met Asn
            740                 745                 750

Ala Gln Val Gly Leu Thr Ser Pro Gly Phe Ile Gly Ala Asp Val Cys
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        755                 760                 765

His Leu Asn Leu His Lys Thr Phe Cys Ile Pro His Gly Gly Gly Gly
    770                 775                 780

Pro Gly Met Gly Pro Ile Gly Val Lys Asn His Leu Ala Pro Phe Leu
785                 790                 795                 800

Pro Ser His Pro Val Ile Pro Thr Gly Gly Ile Pro Gln Pro Glu Lys
                805                 810                 815

Thr Ala Pro Leu Gly Ala Ile Ser Ala Ala Pro Trp Gly Ser Ala Leu
            820                 825                 830

Ile Leu Pro Ile Ser Tyr Thr Tyr Ile Ala Met Met Gly Ser Gly Gly
        835                 840                 845

Leu Thr Asp Ala Ser Lys Ile Ala Ile Leu Asn Ala Asn Tyr Met Ala
    850                 855                 860

Lys Arg Leu Glu Lys His Tyr Pro Val Leu Phe Arg Gly Val Asn Gly
865                 870                 875                 880

Thr Val Ala Arg Glu Phe Ile Ile Asp Leu Arg Gly Phe Lys Asn Thr
                885                 890                 895

Ala Gly Ile Glu Pro Glu Asp Val Ala Lys Arg Leu Met Asp Tyr Gly
            900                 905                 910

Phe His Gly Pro Thr Met Ser Trp Pro Val Pro Gly Thr Leu Met Ile
        915                 920                 925

Glu Pro Thr Glu Ser Glu Ser Lys Ala Glu Leu Asp Arg Phe Cys Asp
    930                 935                 940

Ala Leu Ile Ser Ile Arg Glu Glu Ile Ala Gln Ile Glu Lys Gly Asn
945                 950                 955                 960

Ala Asp Val Gln Asn Asn Val Leu Lys Gly Ala Pro His Pro Pro Ser
                965                 970                 975

Leu Leu Met Ala Asp Thr Trp Lys Lys Pro Tyr Ser Arg Glu Tyr Ala
            980                 985                 990

Ala Phe Pro Ala Pro Trp Leu Arg  Ser Ser Lys Phe Trp  Pro Thr Thr
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Gly Arg  Val Asp Asn Val Tyr  Gly Asp Arg Lys Leu  Val Cys Thr
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tccatctttc tcaaaacatc tctcttactc tcttcgtttt ctgactgcaa cctttttttcc 180

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tctcttccct ttccccctttc atctccaccc ctcgatctgt caaccacacg gcggctttcg 360

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